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THE MODE OF INFECTION AND ETIOLOGY OF EPIDEMIC POLIOMYELITIS.*

By SIMON FLEXNER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

I.

The two problems of uppermost interest in respect to epidemic poliomyelitis are: first, the mode of infection, and second, the nature of the specific microorganism causing the disease.

CORRECTION.

In Volume XIV, Article 21, Page 29, the lower form of the equation should read:

$$= 1.5 D - 1.125 Arg.$$

the validity of the two doctrines be carefully scrutinized and appraised.

Let us examine first the evidence available bearing on the insect conveyance of the disease. This notion was suggested, in the first place, by the seasonal prevalence of epidemic poliomyelitis, which is predominantly a disease of midsummer and early autumn. However, it is not strictly speaking thus narrowly limited in incidence, since cases occur in the spring and even in the winter months, although they are few in number.

In the second place, the notion is supported by the rural character of some epidemics, as well as by the relatively wide distances which separate many of the cases. In these instances, however, the pe-

* Abstract of address delivered before the Philadelphia Pediatric Society, Feb. 9, 1915.

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I.

The two problems of uppermost interest in respect to epidemic poliomyelitis are: first, the mode of infection, and second, the nature of the specific microorganism causing the disease. I propose to deal briefly with these two aspects of the subject.

Two views are entertained regarding the mode of infection; according to one the infectious agent of the disease is communicated by personal contact; according to the other, it is conveyed by the stable fly. The differences involved in these two conceptions are fundamental, and hence the practices looking toward prevention of the malady based on them must be wholly different, if they are to achieve the result desired. For that reason it is imperative that the validity of the two doctrines be carefully scrutinized and appraised.

Let us examine first the evidence available bearing on the insect conveyance of the disease. This notion was suggested, in the first place, by the seasonal prevalence of epidemic poliomyelitis, which is predominantly a disease of midsummer and early autumn. However, it is not strictly speaking thus narrowly limited in incidence, since cases occur in the spring and even in the winter months, although they are few in number.

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cularities are of degree rather than kind. Epidemics of poliomyelitis prevail also in towns and cities, and cases may be closely associated as well as widely separated.

The notion of insect carriage received for a time the support of experimental evidence, without which it would have remained merely a suggestive possibility; and the wide currency which this notion has obtained among the laity depends wholly on an imperfect experimental foundation which has now been largely disproved.

At the Congress of Hygiene and Demography held in Washington in 1912, Dr. M. J. Rosenau of the Harvard Medical School announced, as will be recalled, that he had succeeded in communicating experimental poliomyelitis to several monkeys by permitting stable flies to feed first on monkeys inoculated intracerebrally with the poliomyelitic virus, and then on normal monkeys. Although the studies which he reported were then incomplete and he made his announcement in a judiciously tentative fashion, he expressed the opinion that the flies might carry the infective agent, and also that in conveying it from one human being to another an intervening period of time was necessary, during which the virus underwent some change of development within the insect host. This announcement was followed very quickly by a confirmatory one emanating from Anderson and Frost of the U. S. Public Health laboratory in Washington.

No satisfactory explanation of the successful experiments performed by Rosenau and by Anderson and Frost has thus far been offered, since in no other instance have confirmatory results been obtained. The great importance of the subject led immediately to a repetition of the experiments in several laboratories in this country and in certain laboratories abroad, without yielding a single instance of positive infection. Moreover, Anderson and Frost themselves a little later announced that they had failed subsequently to repeat their earlier successful experiments.

Without pursuing this topic further, we may now turn our attention to the other conception, namely, that infection in poliomyelitis is conveyed through personal contact. In considering that view of the mode of infection it is necessary at the outset to have an understanding of the clinical types of poliomyelitis. So long as the affection

was conceived of as a frankly paralytic disease in all instances, it was impossible to trace the connection of cases one with another, but once it was determined—as was done by Wickman—that epidemic poliomyelitis assumes non-paralytic and ambulant forms, the subject of the mode of infection was opened up to restudy and to a wholly new interpretation.

The proof of the existence of abortive and ambulant forms of poliomyelitis is not clinical merely, but depends also on laboratory findings. The several laboratory findings may be stated briefly to be the following: the detection of changes in the cerebrospinal fluid; the demonstration of neutralizing immunity principles in the blood; and the determination of the presence of the virus of poliomyelitis on the upper respiratory mucous membrane. The changes in the cerebrospinal fluid consist of increased cellular content and the presence of globulin; the two conditions may coexist. The new cells are chiefly lymphocytes. The neutralizing properties of the blood depend on the appearance in the serum of immunity principles absent from the normal blood, which correspond to similar immunity principles arising after a frank attack of paralytic poliomyelitis. The virus of the disease has been detected on the nasal and pharyngeal mucous membranes in such quantity and quality as to make possible the communication of poliomyelitis to monkeys. The unmistakable demonstration, both by clinical observation and laboratory tests, of the existence of abortive and ambulant cases of epidemic poliomyelitis—many of which may and do escape detection—indicates how wide, indiscriminate, and unsuspected the distribution of the virus by personal contact may readily become.

But the facts now in hand carry us beyond the distributing power exercised by the frank and abortive ambulant cases, since it can now be affirmed that epidemic poliomyelitis is one of the diseases in which both healthy and chronic carriers of the microbic agent of infection arise. The few healthy carriers thus far detected consist of persons who have been in very close and intimate contact with persons acutely ill with poliomyelitis; for example, the parents of a paralyzed child. The few instances of chronic carriers now known consist of persons who have recovered from an acute attack, but in whom at the expiration of several months the virus has been detected, by animal inocula-

tion, on the upper respiratory mucous membrane. The two classes last mentioned of potential carriers of the infectious agent add materially to the possibility of wide dissemination of the virus.

It is very important, now that the existence of healthy and chronic carriers of the infectious agent of epidemic poliomyelitis is established, that there should not arise undue concern regarding the dangers of conveying the disease, while the fact should be taken into account in devising measures for the prevention of the conveyance. Possessing as we do at present only the imperfect means of animal inoculation for detecting the virus of poliomyelitis, still it appears that the healthy and chronic carriers are not more numerous in this disease than in many other infections of more common occurrence.

Given, therefore, the possibility of the distribution of the virus being effected by, first, the frankly ill, second, the slightly ill, third, healthy, and fourth, chronic carriers, the striking discrepancy noted between the prevalence of cases in small, sparsely populated rural communities and the more thickly populated towns and cities at once disappears.

To sum up the aspect of the subject concerning the mode of infection, we may now state emphatically that the indications are all in favor of personal communication of the virus. Hence the measures which we seek to put into effect against the introduction and spread of epidemic poliomyelitis should now be based on the conception of the personal factor as paramount, and not on the notion of insect carriage.

II.

I turn now to the question of the nature of the virus or micro-organism causing epidemic poliomyelitis. You will recall that the virus is filterable, that is, it passes through the pores of earthenware filters which exclude under similar conditions the ordinary bacteria. We are now acquainted with a score or so of diseases in man and animals definitely proved or believed to be caused by parasites belonging to this filterable class. It is selfevident that these parasites are very minute; and because of their minuteness it has until recently been doubted whether they had actually been viewed under the microscope.

About two years ago, Dr. Noguchi and I announced that cultures of a very minute organism had been obtained which might be regarded as the possible microbic agent of epidemic poliomyelitis. The cultures were derived from the central nervous organs of human beings and monkeys who had had poliomyelitis. As evidence of the nature of the cultures it could then be stated that experimental poliomyelitis had been produced in monkeys by inoculation of the cultures. We have recently confirmed this result in an interesting manner. The culture employed for inoculation had been isolated about eighteen months before and had gone through a number of generations in artificial mediums. It was cultivated finally in a mixture of ascitic fluid and broth, and the fluid carrying large numbers of the microorganisms—which are of extremely minute size—was injected, in some cases intraspinaly, in others intraperitoneally into rhesus monkeys. The injection produced no immediate effect; indeed, a single injection caused no effect whatever. But when the intraperitoneal and intraspinal injections were repeated three or four times, the animals developed paralysis, and the paralyzed animals showed the peculiar histologic changes of the central nervous system indicative of poliomyelitis. In other words, these experiments showed that a culture of the microorganism mentioned, long removed from the nervous tissues, is capable of causing infection of monkeys, and that by this means the symptoms and lesions of epidemic poliomyelitis are produced.

The fact that several inoculations of the culture were required to cause infection agrees with observations previously made by Lewis and myself, that when a subminimal dose of the usual virus of poliomyelitis is injected into monkeys, no effect is produced; but when the subminimal injections are repeated, paralysis may suddenly supervene. The two sets of observations are, therefore, in accord.

SUMMARY.

The data which I have had the pleasure of laying before you have led me to believe, first, that the microbic agent of epidemic poliomyelitis is present in the nasal and buccal secretions and is carried by persons, not insects, and communicated by them in such manner

as to gain access to the upper respiratory mucous membranes of other persons, among whom a portion, being susceptible to the injurious action of the virus, acquire the infection and develop the disease.

The clinical variety or form of the disease which they develop may be the frankly paralytic, the meningitic, or the abortive and ambulatory in which no severe symptoms whatever appear. But however the persons may be affected, they become potential agents of dissemination of the virus of poliomyelitis, as do a number of healthy persons who have been in intimate contact with those who are ill, and another group of persons who have recovered from an acute attack of poliomyelitis. These several classes of infected or contaminated persons constitute the active means through which the virus is spread and to the control of which sanitary measures designed to prevent epidemics must be directed.

Finally, the virus or microbic agent of epidemic poliomyelitis appears now to have been cultivated and to consist of minute globular bodies, capable of being distinctly viewed under the high powers of the microscope.

THE INFLUENCE OF DIETING UPON THE COURSE OF CANCER.

By PEYTON ROUS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The subject upon which I shall speak to-day—with the great privilege of speaking before this audience—may seem to many of you to have a merely curious interest. Indeed, *a priori* the influence of dieting upon the course of cancer may not seem worth even curiosity. For true it is that throughout time countless people with cancer have eaten and drunk all sorts of things, have been stout or been thin, and yet have ended by dying of their disease. Perhaps, then, it will be well for me to take up some of the general aspects of my subject.

We are accustomed to think and speak of cancer, according to a definition duly remembered, as one of the autonomous new growths. Certainly it is autonomous enough to prove fatal to the patient. Yet you will agree with me that in its general characters the cancer cell is far from showing independence. Apart from its capacity for unlimited growth without differentiation it has almost no distinctive peculiarities. Recent cancer research with animals has made this very evident. So far as we can tell the cancer cell secretes no specific enzyme, gives forth no specific toxin, produces no immunity reaction in the body that can be used with certainty for diagnostic purposes, and has few features that suggest methods of therapeutic attack. The most successful, non-surgical methods of treating cancer are based on quantitative, not on qualitative, differences between the cancer cell and normal cells. The Roentgen ray and radium act selectively on the cancer cell simply because it is somewhat more sensitive than normal cells.

All this being true, is it not possible that, even as regards growth, cancer is less independent than we are given to suppose; and that

bodily conditions which affect normal tissue generally will also affect cancerous tissue? There are certain clinical indications that this is true. Some diseases, notably erysipelas, which profoundly affect the whole organism, may affect a cancer so adversely that it becomes stationary or retrogresses. The observation has been repeatedly made that in withered, old persons cancer grows slowly. And thus one comes to ask whether dieting or special diets may not exert an important influence on cancer. To put a concrete case: When a cancer has been removed that is likely to recur should the patient be fed to plumpness? Is it not possible that by keeping him thin, or underfeeding him so that he loses in weight, a recurrence of the tumor will be delayed?

Manifestly such questions can be answered only by experiments with animals. And now that the status of rat and mouse tumors as true neoplasms is assured this can be readily done. Moreschi was the first to take up such work systematically. He underfed rats so that they lost in weight, and found that grafts of rat tumor had poor success in such animals as compared with full-fed controls. Soon after the appearance of his paper, experiments were begun at the Rockefeller Institute. But though we were able to confirm Moreschi's findings as regards tumor grafts, it proved quite another matter to influence tumors already ensconced and vascularized. A rat carcinoma was found to grow quite as well in rapidly emaciating animals as in those gaining weight on full diet. That a graft introduced into an alien, normal creature should prove less resistant than a tumor which has the organism of the host under its thumb, so to speak, is scarcely surprising. But, unfortunately, it is with the latter condition that one has to do in human beings. In several recent researches this point seems not to have been taken into sufficient account. The investigators have found that special diets exert an adverse influence on cancer grafts in normal hosts. But experiments with tumors already growing have been few and inadequate.

Last year, Dr. Linda Lange and I attempted to determine whether recurrences and metastases of large tumors removed at operation may not behave like grafts in that they are sensitive to dietary influences. Metastases are certainly to be considered as grafts, at one

time in their development, though with the advantage on their side that they are autotransplantations in an animal already the subject of cancer. For our first experiments transplanted tumors were used; but it was soon found that they were not a fit material. I have spoken of the lack of immunity phenomena in animals with cancer. This had reference only to "spontaneous" cancer. In normal animals to which cancer is transferred by grafting there often develops a marked resistance, directed apparently against the growth simply as alien tissue. It may, and frequently does, cause the rapid retrogression of large tumors. It brought much confusion into our experiments, and we came at length to realize that in order to obtain facts for a generalization about spontaneous tumors observations on such tumors would be necessary.

Spontaneous carcinomata are fairly common in the white mouse. Within a short period more than 130 were obtained, all of them tumors of the breast in old females. They were diverse in their histology. For the experiment, half of the tumor animals were underfed so that they lost in weight; half, full-fed; and by a uniform technic the growths were removed save for a small bit. The vessels leading to this bit, which was left to insure a recurrence, were disturbed as little as possible. In each animal two subcutaneous implantations were made of its own tumor. These may be taken to represent in some sort disseminations at the time of operation. The feeding was continued as before and the animals kept under observation for five weeks, a period in the mouse's life equal to about three years in that of a human being—if any such comparison is permissible.

The results were striking. During the five weeks of observation, recurrences appeared in 83 per cent of the full-fed control mice, and in only 41 per cent of those underfed and losing weight at the time of operation. The grafts grew in 68 per cent of the controls and in 41 per cent of the underfed hosts. Evidently restricting the diet has a very marked influence to delay the reappearance of spontaneous mouse tumors.

A word should be said as to the diet. The controls were fed on grain and bread and milk. The experimental animals received Sweet's modification of one of the foods which Mendel and Os-

borne devised in the course of their study of the requirements for normal growth. The food in question is sufficient for the day-to-day needs of rats, but fails to suffice for their growth, because of its lack in certain amino-acids. Rats kept upon it remain healthy but never grow up. Sweet was able to prevent the development of tumor grafts in mice by putting them, previous to inoculation, on his modification of the food. We combined a use of it with underfeeding in the idea that perhaps thus more marked effects might be obtained. But special experiments have shown that our results are to be attributed solely to the underfeeding and resultant loss of weight, and not to the character of the food.

The treatment was drastic. The best results were obtained with animals losing weight rapidly at the time of operation. The average loss in weight at the end of the five weeks of observation was 24 per cent. But as the adult female mouse is a fat, little creature with small bones and an abundant panniculus the emaciation was less than one would suppose.

No cures were effected. In underfed animals put again on full diet a rapid recurrence with growth of the grafts was the rule. In the light of this fact the quiescence of the tumor during the period of dieting can hardly be attributed to processes of resistance. We are inclined to attribute the results in part to direct starvation of the cancer cells, in which they shared with the other cells of the host. There is evidence that in far greater part they were due to a retarded formation of the connective tissue scaffold which furnished blood-vessels and support to the tumor. A very few tumors, and these the most malignant, are able to grow continuously without such a scaffold—to live, so to speak, off the country which they invade. Most require a vascularizing stroma, which is supplied by the host's tissue. In the underfed host, as we have shown by special experiments, proliferative processes take place slowly. Wounds require a long time to heal and an inert foreign body, such as agar-agar, is only tardily organized. It is easy to see why the development of tumors dependent on a supporting stroma would be slower in such animals.

Whether recurrences and the development of metastases in human beings can be influenced by underfeeding is not to be foretold. Im-

portant in this relation is the fact that a small percentage of the tumors in the underfed mice of our experiment—and these the most malignant ones histologically—recurred with a rapidity unexcelled by any of the tumors in full-fed animals. Here the end result of the treatment was a distinct injury, for the host was prematurely emaciated and the development of the growth was unchecked. It is quite probable that the same thing would be true of some human tumors. And to underfeed a cancer patient so that he loses weight is to incur many other dangers. I do not wish, then, to be taken as an advocate for underfeeding in the palliative treatment of cancer. Yet, to judge from the experimental evidence, it might do good in special cases.

PURE CULTIVATION IN VIVO OF VACCINE VIRUS FREE FROM BACTERIA.

BY HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 39 TO 50.

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In spite of much effort no method has, up to the present time, been perfected by which vaccine virus can be propagated in a pure state, free from contaminating bacteria. The method of propagating vaccine virus universally practiced today consists in transmitting the virus from the skin of one calf to that of another. Although the inoculation of the virus is carried out under precautions as strictly aseptic as possible, the fresh product nevertheless yielded by the skin contains a not inconsiderable number of different bacteria derived from the skin surfaces, the air, etc.

The employment of glycerin as an elective germicide against the non-spore-bearing bacteria contained in fresh, or so called "green," vaccine pulp results in a great reduction both in number and variety of the contaminating microorganisms, without at the same time seriously impairing the activity of the vaccine virus (1). After contact with concentrated glycerin, in a refrigerator, ranging from one to three months, the virus usually is freed from most of the bacteria and becomes "ripe" for practical use in vaccination on human beings.

Different samples of the ripe vaccine preparations, as issued from various authorized sources, vary in their activities as well as in their germ contents. Among the organisms which may be encountered in such preparations are the following: streptococci, staphylococci, *Bacillus coli*, *Bacillus welchii*, *Bacillus xerosis*, *Bacillus subtilis*, and some other aerobic and anaerobic bacteria (2). The action of glycerin on bacterial spores, which may also be present in the pulp, is

almost nil. Moreover, the prolonged action of glycerin tends gradually to reduce the activity of the vaccine virus itself, so that the fresh vaccine pulp forfeits some of its efficacy while undergoing the "ripening" process. At the end of the ripening process, which consumes from one to three months, the virus usually still contains a certain number of bacteria; bacteria-free virus is practically never produced by glycerination. But the residue of living bacteria, fortunately, is to all intents and purposes quite harmless and practically to be disregarded. And yet it is apparent that it is not only desirable to eliminate all bacteria from vaccine virus, but the saving of the time lost in "ripening" and the preservation unimpaired by glycerin of the full strength of the virus are also highly important considerations.

HISTORICAL.

The observations of Béraud (3), Weigert (4), Chiari (5, 6), Councilman, Magrath, and Brinkerhoff (7), and others (8) indicated that the variola virus localizes in different viscera and organs; the vaccine virus, on the other hand, according to Calmette and Guérin (9), and von Prowazek and Yamamoto (10), exhibits a high degree of affinity for pavement epithelium. When the vaccine virus is introduced directly into the blood or peritoneal cavity, it lodges in the epithelia of the skin and mucous surfaces, provided they are injured within a few hours of the inoculation, while in a few hours more it has completely disappeared from the circulation or the peritoneum. Should the epithelium not take it up, it is entirely lost.

In order to test the question whether still other epithelial cells take up the virus, Henseval and Convent (11) conducted in 1910 a series of experiments on the testes of rabbits. The virus that they employed contained staphylococci and *Bacillus subtilis*.¹ The effect was to produce some swelling, but no induration of the organ. Five to seven days after inoculation the testes were extirpated, emulsified in 66 per cent. glycerin, and tested on the shaved skin of rabbits. The activity was less than that of the original material. From the first testicular emulsion a second set of rabbits was injected; the swelling and induration produced was greater, but the activity of the emulsion was no stronger. No attempt was made either to free the vaccine of bacteria or to continue the testicular passages. Later, Paschen (12) injected vaccine (its bacterial content is not stated) into the testicle of a rabbit and noted the production of necrosis and cellular infiltration. As early as 1904 Tyzzer (13) attempted to obtain characteristic vaccinia lesions by inoculation of the testis of rabbits and a calf; the result was regarded as an acute inflammatory reaction. Von Prowazek and Miyaji (14) state that vaccine virus injected into the testicle of the rabbit can

¹ The number of contaminating bacteria in the three samples employed was 4, 10, and 25,000 per 0.01 c.c., respectively.

still be detected two days later, and that local necrosis of the tissue appears at the inoculation site. Nothing is said of the bacterial content.

That the testicle of the rabbit affords a favorable site for the multiplication of parasitic microorganisms can be inferred from the experiments of Parodi (15) and later of Uhlenhuth and Mulzer (16) with *Treponema pallidum*, of Nichols (17) with *Treponema pertenue*, and of a host of later workers with *Treponema pallidum* (18). I therefore turned my attention to the cultivation of pure strains of vaccine virus in the rabbit's testicle.

Purification of Vaccine Virus Prior to Cultivation.

At the outset and before describing the details of the experiments on the cultivation of vaccine virus it is necessary to point out a material difference between the employment of the rabbit's testicle for *Treponema pallidum* and for vaccine virus. In the case of the former the multiplication of the spirochætæ takes place slowly, so that the contaminating bacteria which are carried into the testicle with the inoculated fragments of syphilitic tissue may in time be suppressed by the bactericidal action of the vigorous tissues. On that account pure strains of the pallidum may be thus developed after a few passages. Vaccine virus, on the other hand, multiplies quickly, and the inflammatory reaction and necrosis of tissue which ensue quickly suppress the bactericidal process of the testicular tissues and place them in a favorable state for bacterial development. On that account the virus must first be freed from the bacterial contamination by other means.

Preliminary Purification.—Several antiseptic or disinfecting substances may be employed for reducing or removing contaminating bacteria. Glycerin is universally employed in practice to reduce the number, because of the small effect which it exerts on the virus itself. At low temperatures the antiseptic action is too weak to eliminate all bacteria, but at 37° C. in 60 per cent. strength all bacteria except spore-bearing species may be destroyed in a few days. Hence it is suitable for the purification when the virus is devoid of sporogenous bacteria. Addition of 1 per cent. phenol alone or 1 per cent. phenol and 60 per cent. glycerin both to the emulsion brings about a quicker elimination of bacteria, but this is accompanied by a more rapid deterioration of the virus itself. The same is true with the addition of one part per thousand of oil of cloves to 60 per cent. glycerin.

Recently Fornet (19), who has experimented with cultivation *in vitro* of vaccine virus, has recommended ether for removing the bacteria which do not possess spores. An emulsion of the virus can be freed of all bacteria except sporogenous ones by being shaken at room temperature for forty-eight hours with an excess of ether. The activity of the virus is, however, considerably diminished by ether treatment. By the ether method a vaccine pulp devoid of spore-bearing bacteria obtained as follows was secured free of all bacteria, and hence was suitable for cultivation in a pure state *in vivo*.

A sample² of glycerinated virus free from sporogenous bacteria was incubated at 37° C. for two days or longer, in order to destroy practically all the bacteria still present in it. The skin on the dorsal side of a rabbit was shaved and thoroughly cleansed with soap and rinsed with sterile distilled water. It was again shaved closely, after which the glycerinated virus was applied, in such strength as to produce separate eruptions. The vaccinated surface is protected from contamination by means of a sterile bandage. On the fourth or fifth day the bandage was removed and the surface washed first with absolute alcohol and then with ether. Several vesicles were selected and each was cleansed with 5 per cent. lysol solution and washed alternately with absolute alcohol and ether on sterile gauze. The eruptions were scraped with the edge of a sharp scalpel and the scrapings emulsified in several cubic centimeters of sterile saline solution. The emulsion was mixed with several volumes of ether and shaken in a sealed vessel for varying periods of time at room temperature. Samples were removed at the expiration of 1, 2, 4, 8, 12, 24, and 48 hours, from each of which cultures were prepared. When spores are absent sterility is usually obtained in twelve hours. The vaccinal activity falls at times to one-fifth of the original strength, but the characteristic properties remain unaltered.

Testicular Cultivation in Rabbits.

The vaccine emulsion so prepared is employed for the intratesticular inoculation of rabbits. Rabbits with well developed testicles should be chosen. After ether anesthesia an assistant holds the animal and fixes the testicles to prevent their withdrawal into the peritoneal cavity. The scrotal skin which is tightly stretched over the testicle is sterilized with 5 per cent. lysol solution and then painted with tincture of iodine. The operator next inserts the needle of a

² Several samples of the vaccine virus employed in the present investigation were furnished me by Dr. F. S. Fielder, Assistant Director of the Vaccine Laboratory of the Department of Health of the City of New York, to whom I wish to express my gratitude.

sterile syringe containing a 1 to 10 or 1 to 20 dilution of the emulsion into the testicular parenchyma along the long axis. The point of the needle is prevented from passing through the parenchyma to the tunica vaginalis. The contents are now gently forced out of the syringe into the organ at different regions by turning the direction of the needle. About one cubic centimeter of emulsion is injected into a testicle weighing from two to three grams. The organ is gently massaged to distribute the virus throughout the entire organ. The operation is practically painless.

The method just described is employed for the inoculation of the testicular strains of the virus, in which case an emulsion of testicle previously inoculated with the virus is used. The stock emulsion is prepared by grinding up the aseptically removed organs with sterile saline or 60 per cent. glycerin solution in the proportion of one gram of the tissue to two or three cubic centimeters of the fluid and any dilution of it; about 1 to 10 or 1 to 20 in saline is prepared for the purpose of the injection.

The testing for bacteria in the emulsion is an important point. Cultures are set up with plain and glucose bouillon, ascitic fluid with and without bouillon, and ascitic fluid with a piece of fresh sterile rabbit kidney with and without a layer of sterile paraffin oil. The cultures are incubated at 37° C. for three to four days, and then subcultures on plain and ascitic glucose slant agar, deep layer glucose agar, and ascitic tissue deep layer agar are made.³ Film preparations stained by Gram or by Giemsa are also examined under the microscope, both from the cultures and from the testicles removed from the animal. In order to avoid occasional bacterial infection it has been found well to inoculate both testicles of each animal. The skin may be vaccinated at the same time. It is, moreover, advisable to use at least two rabbits for each transfer, because sometimes a rabbit reacts poorly to the vaccinal inoculation of both skin and testicle; but once the virulence of the virus has reached a certain height this precaution is no longer necessary.

³ In the latter part of the present work initial culture in tissue bouillon and subcultures in glucose agar (deep) and plain agar (plate) were found to be sufficient.

Results of Experiments with Rabbits.

As the accompanying table shows, the passage of the testicular virus from animal to animal offered some difficulty until it had been carried on for a number of generations. In this experiment success was not assured until the virus had been transferred about ten times. That this result was due to increase in virulence of the virus in the later generations may be deduced from the greater activity manifested when it was tested upon the skin, as well as from the greater severity of the reactions in the testicles themselves (table I).

TABLE I.

Date of transfer.	Generation.	Results.	Date of transfer.	Generation.	Results.
1914					
Jan. 29-Feb. 2.....	1	+	Sept. 28-Oct. 3.....	32	++
Feb. 2-9.....	2	+	Oct. 3-7.....	33	++
Feb. 9-14.....	3	+	Oct. 7-12.....	34	++
Feb. 14-22.....	4	+	Oct. 12-16.....	35	++
Feb. 23-27.....	5	++ ⁴	Oct. 16-20.....	36	++
Mar. 10-16.....	6	++ ⁵	Oct. 20-25.....	37	+++
Apr. 22-25.....	7	++ ⁶	Oct. 25-29.....	38	+++
May 1-6.....	8	+	Oct. 29-Nov. 3.....	39	+++
May 25-29.....	9	+	Nov. 3-7.....	40	+++
May 29-June 3.....	10	++	Nov. 7-12.....	41	+++
June 3-6.....	11	++	Nov. 12-16.....	42	+++
June 8-12.....	12	++	Nov. 16-21.....	43	+++
June 12-15.....	13	++ ⁷	Nov. 21-26.....	44	+++
June 18-25.....	14	++	Nov. 26-30.....	45	+++
June 25-July 2.....	15	++	Nov. 30-Dec. 5.....	46	+++
July 2-8.....	16	++	Dec. 5-9.....	47	+++
July 8-13.....	17	++	Dec. 9-13.....	48	+++
July 13-16.....	18	++	Dec. 13-16.....	49	+++
July 17-20.....	19	++	Dec. 16-20.....	50	+++
July 20-23.....	20	++	Dec. 20-24.....	51	+++
July 26-30.....	21	++	Dec. 27-31.....	52	+++
July 30-Aug. 3.....	22	++	1915		
Aug. 4-7.....	23	++	Jan. 4-8.....	53	+++
Aug. 11-17.....	24	++	Jan. 8-12.....	54	+++
Aug. 17-22.....	25	++	Jan. 15-19.....	55	+++
Sept. 1-4.....	26	++	Jan. 23-27.....	56	+++
Sept. 7-10.....	27	++	Jan. 31-Feb. 4.....	57	+++
Sept. 12-16.....	28	++	Feb. 8-12.....	58	+++
Sept. 16-20.....	29	++	Feb. 16-20.....	59	+++
Sept. 21-24.....	30	++	Feb. 24-28.....	60	+++
Sept. 24-28.....	31	++			

⁴ From this material two further generations were carried on and then lost, so it was necessary to come back to this generation and to start again.

⁵ From this two generations were carried on and lost, necessitating a return.

⁶ Only one out of several rabbits gave good results.

⁷ Two rabbits were inoculated with this material, but only one gave a satisfactory result.

The testicular strain indicated in the table has passed through sixty successive generations within twelve months. It is interesting to find that considerable resistance to testicular adaptation was exhibited at the seventh transfer, after which no serious obstacle was met in carrying on the passages.

Testicular Vaccinal Processes in Rabbits.

In order to follow the course of the vaccinal processes in the testicular tissue the following experiments were carried out.

Thirteen male rabbits were inoculated into each testicle with 1 c.c. of a 1:20 dilution of the saline testicular emulsion derived from the rabbit inoculated with the thirtieth generation of the testicular strain and castrated under ether anesthesia on the fourth day.

The testicles of these animals were removed one after the other and tested successively for activity on the skin, cornea, and testicles of normal rabbits every twenty-four hours, over a period of eighteen days, and then after 3, 4, 5, and 8 weeks.

Table II shows the results obtained.

During the first twenty-four hours the testicle presents little change, except that microscopic foci of infiltration of polynuclear leucocytes and exudate are observed in the interstitial spaces (figure 32, compare with normal, figures 30 and 31). At the end of forty-eight hours the swelling and induration of the organ begin to increase rapidly and the testicle becomes congested and edematous (figure 2). The content of the virus, which was almost zero after twenty-four hours, now reaches about 100 times that found at the end of the first day. When examined in sections an enormous leucocytic infiltration is seen in the interstitial tissues, and some leucocytes are contained in tubules (figure 33). The testicular cells are hydropic and fill up the tubular lumen. At the end of three days the infiltration has increased in intensity and extent (figures 3 and 18, compare with normal, figure 17), and the vaccinal activity has risen to at least 300 times that present twenty-four hours and about three times that present forty-eight hours after the inoculation.

The external changes present in the four day specimen (figures 4 and 34) resemble those occurring in the three day specimen, except that the testicle is more compact and less elastic and the amount of

TABLE II.

Testicles removed after	Gross appearance of specimens.	Tests for activity of emulsions on rabbits.			
		Dilution of emulsion.	Skin.	Cornea	Testicle.
24 hrs.	Almost no swelling; traumatic hemorrhages	1 : 10	1 eruption	—	+
		1 : 100	—		—
		1 : 1,000	—		—
48 hrs.	Vascular injection, edema, and moderate induration (figure 2)	1 : 10	++	++	++
		1 : 100	+	—	
		1 : 1,000	<+	—	
3 dys.	Marked induration, great congestion, greyish mottling, edema (figure 3)	1 : 10,000	—	—	—
		1 : 10	++	++	++
		1 : 100	++	+	
4 dys.	Severe congestion, edema, induration and swelling; numerous yellowish grey spots on the organ (figure 4)	1 : 1,000	+	—	
		1 : 10,000	—	—	—
		1 : 10	Confluent	++	++
5 dys.	Similar to the last (figure 1)	1 : 100	+++	+	
		1 : 1,000	++	+	
		1 : 10,000	<+	—	++
5 dys.	Similar to the last (figure 5)	1 : 10	Confluent	++	++
		1 : 100	Confluent	++	
		1 : 1,000	++	+	
5 dys.	Extensive hemorrhages; otherwise similar to the last	1 : 10,000	+	?	++
		1 : 10	Confluent	++	++
		1 : 100	++	+	
6 dys.	Marked induration, but somewhat less than on the previous day. Less edema (figure 6)	1 : 1,000	+	—	
		1 : 10,000	—	—	+
		1 : 10	Confluent	++	++
7 dys.	Induration somewhat less than on the preceding day. Yellowish grey specks prominent on the surface (figure 7)	1 : 100	++	+	
		1 : 1,000	+	—	
		1 : 10,000	—	—	+
8 dys.	General induration disappearing; organ flabby; disseminated yellowish grey spots (figure 8)	1 : 10	+	++	++
		1 : 100	<+	—	
		1 : 1,000	—	—	
9 dys.	Induration gone; soft and pale, slightly below original volume; some focal infiltration (figure 9)	1 : 10,000	—	—	—
		1 : 10	+	+	++
		1 : 100	<+	—	
10 dys.	Induration gone; general atrophy; some focal induration (figure 10)	1 : 1,000	<+	—	
		1 : 10,000	—	—	—
		1 : 10	+	+	++
11 dys.	Marked reduction in size; no induration (figure 11)	1 : 100	<+	—	
		1 : 1,000	—	—	—
		1 : 10,000	—	—	—
12 dys.	No induration, soft and pale, much smaller than original (figure 12)	1 : 10	+	+	+
		1 : 100	—	—	
		1 : 1,000	—	—	
		1 : 10,000	—	—	—

TABLE II.—*Concluded.*

Testicles removed after	Gross appearance of specimens.	Tests for activity of emulsions on rabbits.			
		Dilution of emulsion.	Skin.	Cornea.	Testicle.
13 dys.	Normal in size, pale and soft; some greyish spots	1 : 2	+	+	
		1 : 10	< +		+
		1 : 20	—	—	
		1 : 100	—		
14 dys.	Similar to the last	1 : 2	—	—	
		1 : 10	—		+
		1 : 20	—		
15 dys.	Small, pale, flabby; few greyish foci	1 : 2	+	+	
		1 : 10	< +	—	+
		1 : 20	< +	—	
16 dys.	Similar to the last	1 : 2	+	+	
		1 : 10	< +	—	+
		1 : 20	< +	—	
17 dys.	Marked atrophy	1 : 2	—	—	+
		1 : 10	—	—	
		1 : 20	—	—	
18 dys.	Similar to the last	1 : 2	—	—	—
		1 : 10	—	—	—
		1 : 20	—	—	
21 dys.	Small, fibrous; few minute foci of infiltration	1 : 2	< +	+	+
28 dys.	Similar to the last	1 : 10	—	—	
		1 : 2	eruption	—	+
35 dys.	Marked atrophy; small area of necrotic tissue present	1 : 10	—	—	
		1 : 2	—	—	—
56 dys.	One testicle reduced to a small fibrous mass; the other less atrophied	1 : 10	—	—	—
		1 : 2	—	—	—

exudate in the tunica vaginalis and in the testicular tissue itself is more copious. The color of the testis has become purplish red, spotted here and there with irregular yellowish areas of different dimensions (figures 13 to 16). The organ is easily torn. The sections show numerous groups of several tubules each, which have lost the property of taking up the basic stain, indicating a total necrosis of the structures (figures 19 and 36). These changes are not universal, since areas exist in which no apparent serious alteration has taken place. At this stage the vaccinal power is nearly three times as great as it was at the end of three days, and at least 1,000 times as great as at the end of the twenty-four hour period. The changes in the five day specimen are about the same as those of the four day specimen. The vaccinal activity seems now to have attained its maximum height, since almost confluent eruptions on the skin of rabbits are yielded in dilutions of 1 to 1,000. The sections indicate beginning disintegration of the leucocytic elements and of

affected tubular cells (figures 5, 29, 29 a, and 35; compare with normal, figure 28).

At the expiration of six days the testicle has become of softer consistency (figure 6), and the edema and cellular infiltration have begun to recede. Microscopically foci of small round cells in the infiltrated areas and fibrin masses are distinguished (figures 37 and 38). The vaccinal activity is similar to that of the five day specimen. After seven days the resolution of the infiltration proceeds more rapidly (figures 7 and 20), while the vaccinal activity is less than that of the preceding day. From now on the testicles diminish rapidly in volume, so that the ten day specimen is below normal in size (figures 8, 9, 10, 21, and 22). The organs are now pale and of soft texture. The activity of the virus is much less than it was at the end of seven days. At the end of eleven days it is still weaker. From twelve to eighteen days the shrinkage of the organ continues and the sections show loss of testicular cells (figures 11, 12, 23 to 27, and 39 to 44) and collapse of the tubules. The virus has now disappeared wholly or almost wholly, and in some instances no vaccinal effect could be obtained in any concentration. Hence this period may be regarded as that of elimination of the virus, while the exact moment of disappearance probably depends upon the quantity of the virus originally developed and the degree of immunity displayed. At later periods, namely after five weeks, no virus could be detected, while the atrophy of the testicular parenchyma may be complete, so that a fibrous mass containing a few unusually thin tubules, lined with a single layer of epithelial cells may alone remain (figures 27 and 45).

The tunica vaginalis is often intensely infiltrated with polynuclear leucocytes (figure 46) and contains a considerable amount of the virus; apparently the virus multiplies here as it does in other epithelial cells. The epididymis shows a slight infiltration only during the early period.

Spermatogenesis ceases quickly and the various sperm cells quickly degenerate under the influence of the vaccinal process.

Effects of the Testicular Vaccinal Strain on the Skin and Cornea.

The testicular strains of the virus were employed to inoculate the shaved skin and scarified corneal surfaces of rabbits. The effects of the vaccinations so carried out may be followed in table III. They will be recognized as characteristic of the effects produced by active vaccine virus as usually prepared in the calf. The microscopical features of the process are also typical, including the presence of the Guarnieri, or vaccine, bodies so called (figures 49 and 50).

TABLE III.

At the end of	Average course in rabbits.		
	Skin.	Cornea.	Temperature. ^a
24 hrs.	Diffuse reddening	Slight swelling	38.9°
48 hrs.	Mottled reddish areas	Slight turbidity	39.5°
3 dys.	Fairly defined flat erythema, in part confluent	Distinct turbidity	40.0°
4 dys.	In part confluent, mostly discrete raised papules with induration and areola	Advancing in degree and extent	40.8°
5 dys.	Distinct raised round vesicles with areola	Sometimes ulceration	40.2°
6 dys.	Development into pustular eruptions	Ulceration	39.8°
7 dys.	Pustules with scanty content; tendency to dry up	No change	38.8°
8 dys.	Inflammatory processes disappearing	Ulcer persists	38.9°
9 dys.	Stage of crust formation and desquamation	Ulcer persists	38.7°
10 dys.	Stage of crust formation and desquamation	Ulcer persists	38.8°

Effects of Varying Concentration of Testicular Virus upon the Skin, Cornea, and Testicle.

In order to determine the sensitiveness of the skin, cornea, and testicle to vaccine virus, emulsions of the testicular virus of different concentrations were prepared and inoculated into the several parts mentioned.

For this purpose a specimen of testicle representing the thirty-first passage or generation was employed. The dilutions in sterile saline were as follows: 1 to 10, 1 to 100, 1 to 300, 1 to 1,000, 1 to 3,000, 1 to 10,000, 1 to 30,000, 1 to 100,000. The skin and cornea were

^a Average from fifteen rabbits.

inoculated in the usual manner, and 0.5 of a cubic centimeter was injected into the testicle.

The result is summarized in table IV and may be stated as follows: Up to a dilution of 1 to 300 the testicular emulsion causes marked vaccinal reactions in the skin, cornea, and testis. The skin surface still reacts slightly at the 1 to 1,000 dilution, but the cornea does not. Beyond the 1 to 1,000 dilution the skin reacts no longer, while the testicle continues to react even to dilutions of 1 to 100,000, the limit of the experiment. However, dilutions of 1 to 3,000 and higher retarded somewhat the testicular reaction, which in the end

TABLE IV.

Dilution of emulsion.	Reactions following the inoculation of different dilutions of the emulsion, as indicated in the first column.			Tests of activity of emulsions prepared from testicles* which had been injected with different dilutions.			
	Skin.	Cornea.	Testicle.	Dilution of emulsion of each testicle.	Skin.	Cornea.	Testicle.
1 : 10	+++	+	3 dys. already marked induration and edema	1 : 100	+	+	++
1 : 100	++	+	3 dys. already marked induration and edema	1 : 1,000	+		++
1 : 300	++	+	3 dys. already marked induration and edema	1 : 100	++	++	++
1 : 1,000	+	—	3 dys. already marked induration and edema	1 : 1,000	+		++
1 : 3,000	—	—	3 dys. only slight induration; 5 dys. marked increase	1 : 100	++	++	++
1 : 10,000	—	—	3 dys. only slight induration; 5 dys. marked increase	1 : 1,000	+		++
1 : 30,000	—	—	3 dys. only slight induration; 5 dys. marked increase	1 : 100	++	++	++
1 : 100,000	—	—	3 dys. only slight induration; 5 dys. marked increase	1 : 1,000	+		++

was quite identical with that produced by the stronger concentration. In an experiment made somewhat later and after the testicular strain of the virus had been intensified by repeated passages, a skin reaction of small degree but of characteristic kind could be elicited in the 1 to 100,000 dilution.

This experiment serves also to bring out distinctly the fact of

* The testicles were removed from the animals on the sixth day after the inoculation.

the rapid multiplication of the virus in the testicle of rabbits, and is shown in the second half of table IV. The vaccinal effect upon the skin, cornea, and testicle became essentially the same in kind and degree, irrespective of the concentration of the virus emulsion injected originally into the testicle. At the expiration of six days the testicles inoculated with virus in the 1 to 100,000 dilution yielded a virus emulsion as active as the original testicular material employed for preparing the stock emulsion. The rapidity and ease of multiplication of the virus within the testicle is well illustrated by this experiment. The causes of the small variations in effect as indicated in the table as occurring between emulsions of testicles receiving the higher and lower dilutions of virus are not at once apparent. Possibly certain rabbits restrain the multiplication of the virus more than others; possibly concomitant immunity reactions come into play.

Testicular Cultivation in Bulls.

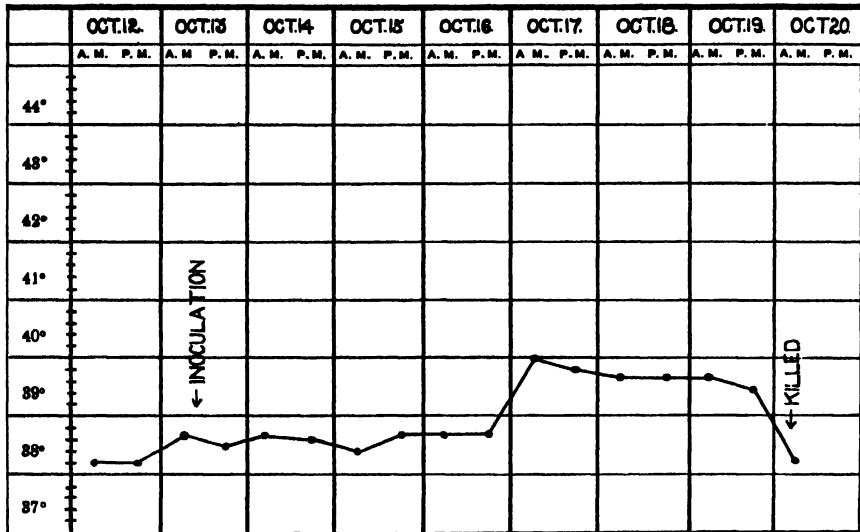
Experiments were next performed on the testicles of young bulls, with the view of ascertaining whether this species of animal would be suitable for use, both from the quantitative and qualitative relation of the virus, since it has long been customary to produce vaccine in the skin of this species.

Bull 1.—Weight, 750 pounds. The scrotal skin was shaved, washed with soap, and sterilized by sublimate alcohol. The vaccine virus employed for the injection consisted of testicular emulsion derived from the testicles of two rabbits which had been inoculated with the 30th generation of the testicular strain and castrated at the end of seven days. This emulsion produced a fairly thick eruption on the skin of rabbits in the dilution of 1:100. On Oct. 13, 1914, 10 c.c. of the emulsion diluted 1:100 were injected into the right, and 15 c.c. into the left testicle under aseptic precautions.

The testicles showed signs of swelling on the third day (forty-eight hours), and increased further in size and density up to the end of six days. The left testicle was much more swollen than the right. The temperature remained normal (38.7° to 38.9°) until the end of the fifth day, when it rose to 40.6°, 40.1° (6th day), and remained at 40.4° as late as the end of the sixth day. On the morning of the eighth day the temperature was 38.7° (text-figure 1).

The animal was slaughtered and the testicles were removed under aseptic precautions on the eighth day. The subcutaneous tissue and the tunica vaginalis were edematous and hemorrhagic, especially along the needle track. The testicles themselves were also edematous and showed a mass of coagulated blood at the site where a blood vessel must have been injured by the needle puncture.

Compared with the normal the vaccinated testicles were firmer and more pinkish in color, besides showing numerous ecchymoses. The left testicle was more altered than the right; the former weighed 310 and the latter 250 gm.



TEXT-FIG. I. Bull 1.

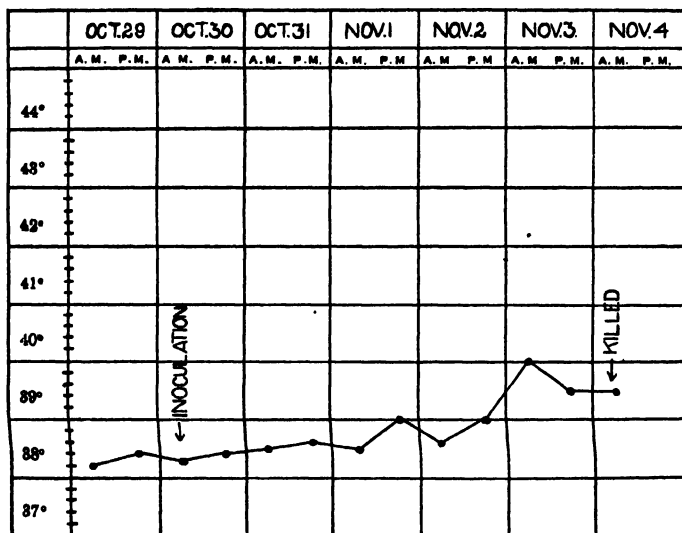
Emulsions in sterile saline solution were made with the tissues immediately about the point of insertion of the needle and also of portions remote from the site of injection. The tests for activity were made upon the shaved skin, cornea, and testicles of rabbits.

TABLE V.

Bull 1.	Tests on rabbits.			
	Dilution of emulsion.	Skin.	Cornea.	Testicle.
Emulsion of tissue around site of injection.	1 : 10	+++	++	++
	1 : 100	+	+	++
	1 : 1,000	<+	-	++
	1 : 10,000	-		++
Emulsion of tissue remote from site of injection. .	1 : 10	++	++	++
	1 : 100	+	-	++
	1 : 1,000	-		-
	1 : 10,000	-		-
Exudate in tunica vaginalis.	Undiluted	-	-	-
	1 : 10	-	-	-

The exudate contained in the tunica vaginalis was likewise tested. Both exudate and emulsions were found sterile for bacteria. Table V summarizes the results, which show that the virus multiplied to a much smaller extent in the testicular tissues of the bull than is the rule in the testicle of the rabbit. That the virus multiplied in some degree is highly probable; and that the edematous fluid in the tunica vaginalis was devoid of virus is an interesting point.

Bull 2.—Weight, 680 pounds. Oct. 30, 1914. The left testicle was injected at four different sites in order to distribute the virus widely with a 1:100 dilution of rabbit testicular virus in the thirty-third generation, the total quantity inoculated amounting to 20 c.c. At the same time the skin of the ventral surface below the navel was shaved, scarified, and inoculated with some of the same emulsion. The inoculated testicle after forty-eight hours was tense on palpation, distinctly swollen, and indurated. The swelling and induration increased during the next three days. The skin showed distinct papules three days after the inoculation, which became typical pustules two days later (figure 53). The temperature rose to 40° to 40.7° on the fourth day, and was 40° on the sixth day. The bull was slaughtered on the sixth day and the testicles were aseptically removed (text-figure 2).



TEXT-FIG. 2. Bull 2.

The scrotum was found to be edematous and infiltrated, but no adhesion was present between the tunica and the testicular surface. The moderate amount of serous and hemorrhagic exudate proved to be sterile for bacteria. Upon section

serous exudate oozed freely from the testicle. The points of injection could be detected by the presence of numerous minute hemorrhages within areas which were distinctly firmer than the surrounding parts. Coagula of blood occurred here and there (figures 54 and 55). The uninoculated right testicle showed no change. The weight of the left testicle was 229 and that of the right 199 gm.

Emulsions were prepared both from the tissues immediately about the sites of injection and from parts remote from them. The tests were made on the skin, cornea, and testicles of rabbits.

TABLE VI.

Bull 2.	Tests on rabbits.			
	Dilution of emulsion.	Skin.	Cornea.	Testicle.
Emulsion of tissue around zone of injection.	1 : 10	Confluent	++	++
	1 : 100	++	+	++
	1 : 1,000	+		++
	1 : 10,000	<+		++
Emulsion of tissue remote from site of injection.....	1 : 10	++	++	++
	1 : 100	+	+	++
	1 : 1,000	<+		+
	1 : 10,000	—		—
Exudate in tunica vaginalis	Undiluted	<+	+	+
	1 : 30	—	—	—

Table VI, which summarizes the results, indicates that the tissues about the sites of inoculation contain an amount of the virus about equal to that of rabbit's testicles inoculated with similar material, and that the degree of activity possessed by the two testicular strains is about the same. On the other hand, the emulsion made from the remote portions of the testicle produced far less effect, although it was still active up to the dilution of at least 1 to 1,000. The exudate in the tunica contained a trace of the virus, but this may have been due to a contamination of this exudate in the opening of the testicle.

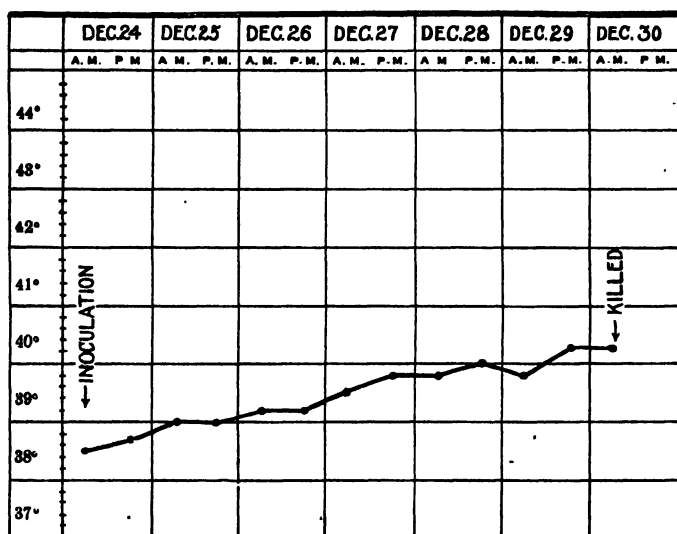
Bull 3.—Weight, 720 pounds. Nov. 11, 1914. Both testicles were inoculated with an emulsion of a testicular strain obtained from a rabbit inoculated with the testicular strain of bull 2. The emulsion was, however, contaminated with a *xerosis* bacillus. The local and general reactions were similar to those described in the previous two bulls. In spite of the admixture of *xerosis* bacilli with the virus the local reaction was not different from those following the injection of a pure virus. The animal was slaughtered on the ninth day after injection.

The testicular tissues were edematous, showed several well localized areas of

a greyish white color, and some hemorrhages caused by the trauma of the needle. The tissues were not softened. Cultures made from the emulsions of the testicles showed the presence of a few *xerosis* bacilli, but no other bacteria.

The vaccinal activity as tested on the rabbit showed that it was much weaker than that of bull 2.

Bull 4.—Weight, 140 pounds. Dec. 24, 1914. 4 c.c. of a 1:40 dilution of an emulsion of the fiftieth generation of testicular rabbit virus were injected into the left, and 3 c.c. of a 1:10 dilution of the testicular virus from bull 2 were injected into the right testicle. The organs were examined every twenty-four hours, but no particular difference was noticed on the two sides. Both showed marked induration within thirty hours. On the fifth day the swelling appeared to have reached the maximal stage. The temperature rose after three days and reached 40.8° on the sixth day. The animal was slaughtered at the end of six days (text-figure 3).



TEXT-FIG. 3. Bull 4.

The testicles had increased in volume, especially the one on the left side injected with the bull-rabbit strain; they were markedly edematous and congested. On section numerous greyish yellow specks were seen to be scattered throughout the organ, and were especially numerous along the sites of injection. Similar greyish yellow areas, it will be recalled, occur almost constantly in the inoculated rabbit's testicles.

The emulsions were made from both testicles separately and tested for their vaccinal activity, together with the controls of the standard glycerinated vaccine issued by the Department of Health of New

York City. The testicular emulsions were free of bacteria; the Health Department vaccine, of course, was not.

TABLE VII.

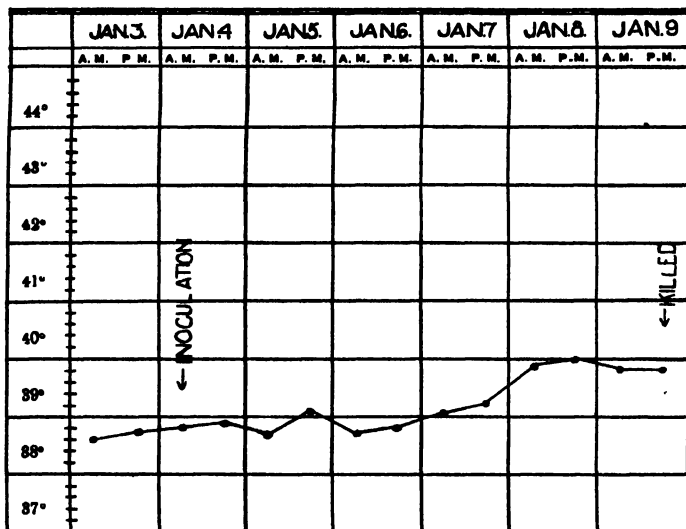
Bull 4.	Dilution of emulsion.	Tests on rabbits.			Tests on calves.	
		Skin.	Cornea.	Testicle.	Skin.	
Emulsion of testicle inoculated with bull-rabbit testicular strain	1 : 10	Confluent	++	++	Confluent.	
	1 : 100	Confluent	+	++	++	
	1 : 1,000	+	±	++	+	
	1 : 10,000	< +	-	++	< +	
Emulsion of testicle inoculated with bull-rabbit testicular strain no. 2	1 : 10	Confluent	++	++	Confluent.	
	1 : 100	Confluent	++	++	Almost confluent.	
	1 : 1,000	++	+	++	++	
	1 : 10,000	+	-	++	< +	
Regular vaccine as controls	N. Y. D. H. standard vaccine 1st sample	1 : 10	Confluent	++	++	Confluent.
		1 : 100	Confluent	++	++	Almost confluent.
		1 : 1,000	++	+	+	++
		1 : 10,000	< +	-	< < +	< +
	N. Y. D. H. standard vaccine 2d sample	1 : 10	Confluent	++	++	Confluent.
		1 : 100	++	+	+	++
		1 : 1,000	+	-	-	+
		1 : 10,000	-	-	-	-

The above experiments (table VII) demonstrate that a strong bacteria-free vaccine virus can be produced in the testicular tissues of the young bull by injecting the testicular strain derived from a bull or from a rabbit, and they also show that the vaccinal activity reaches the same strength as that possessed by a standard virus propagated on the skin of a calf. Moreover, no differences were noted in the type and course of the vaccinal processes as produced in the skin of rabbits and calves by the vaccines from the several sources. The only distinctions are quantitative ones, since the skin virus is more quickly diluted beyond effective strength than the testicular virus.

The next experiment was devised to compare the effect of the testicular inoculation of a testicular and a skin strain of vaccine, respectively, into the same calf.

Bull 5.—Weight, 160 pounds. Jan. 4, 1915. 3 c.c. of a 1 : 10 dilution of an emulsion of the testicular strain derived from bull 4 were injected into the right, and 3 c.c. of a 1 : 10 dilution of the New York City Department of Health standard vaccine into the left testicle. The next day the swelling and injection of the right testicle were marked, while the left was far less altered. This difference

became more pronounced, and on the sixth day only the right side presented the typical reaction, swelling, and induration previously noted, while the left was but slightly enlarged. The animal was slaughtered at the end of five and one-half days (text-figure 4).



TEXT-FIG. 4. Bull 5.

Upon exposing the testicles it was found that the left showed infiltration in small amount and only about the site of the injection, while the right was edematous throughout, greatly enlarged, and dotted with numerous minute foci of greyish yellow appearance. While the consistency of the left testicle which weighed 6.5 gm. was about normal, that of the right, which weighed 8 gm., was diminished.

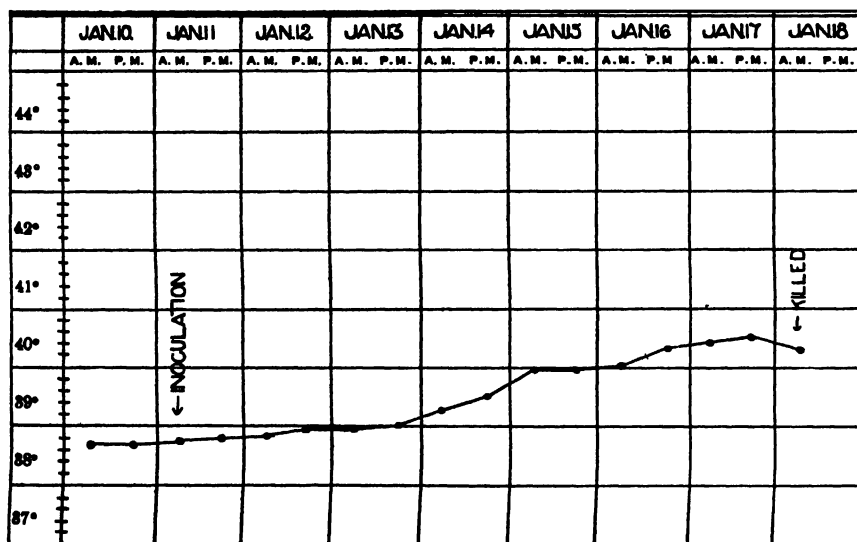
The vaccinal strength of both testicles was tested on the rabbit.

TABLE VIII.

Bull 5.	Tests on rabbits.			
	Dilution of emulsion.	Skin.	Cornea.	Testicle.
Emulsion of testicle inoculated with calf testicular strain no. 4	1 : 10	Confluent	++	++
	1 : 100	++	++	++
	1 : 1,000	+	-	++
	1 : 10,000	-	-	++
Emulsion of testicle inoculated with skin strain (N. Y. D. H.)	1 : 10	+	+	+
	1 : 100	<+	-	-
	1 : 1,000	-		-
	1 : 10,000	-		-

As table VIII indicates, the local reaction and the vaccinal activity were more marked in the case of the testicular than of the skin strain, from which it may be inferred that adaptation of the strain to the testicle is necessary as a prerequisite to the production by this means of strong vaccine.

Bull 6.—Weight, 225 pounds. Jan. 11, 1915. The left testicle was injected with an emulsion of the testicle that had been inoculated with the standard skin strain of vaccine, as mentioned above, and the right testicle with an emulsion of the testicle previously inoculated with a testicular strain. The local and general reactions were similar to those observed and described, except that the left testicle, weighing 9 gm., showed far less change than the right, which weighed 12.5 gm.



TEXT-FIG. 5. Bull 6.

(figures 56 and 57). The bull was slaughtered at the end of five and one-half days, and the vaccinal activity of each testicle was tested on the rabbit (table IX and text-figure 5).

Histological Changes.—The histological changes observed in the testicles of bulls inoculated with testicular strains of vaccine virus are similar to those enumerated in these organs of the rabbit. An acute inflammatory reaction arises, in which many polymorphonuclear leucocytes invade the interstitial tissues together with a serous exudate; the adjacent tubules are compressed by pressure of the exu-

TABLE IX.

Bull 6.	Tests on rabbits.		
	Dilution of emulsion.	Skin.	Cornea.
Emulsion of left testicle inoculated with skin strain.	1 : 10	+	+
	1 : 100	< +	-
	1 : 1,000	-	
	1 : 10,000	-	
Emulsion of right testicle inoculated with testicular strain	1 : 10	+++	++
	1 : 100	++	+
	1 : 1,000	+	
	1 : 10,000	-	

date, which also invades them, and the epithelial cells degenerate. The reaction is multifocal and affects parts remote from the site of inoculation, although the severer effects occur adjacent to the inoculation. Hemorrhages occur also (figures 57 to 66).

Identity of the Skin and Testicular Strains of Vaccine Virus.

It may now be considered as established that vaccine virus derived from the specific skin lesions is capable of multiplying indefinitely in a pure state within the testis of the rabbit and the bull. There would appear to be no necessity for restoring the virus to skin surfaces in order to continue the propagation in the testicles. The testicular epithelium, once the virus is adapted to it, seems to provide as suitable a medium for its multiplication as the skin epithelium. Moreover, in order that the virus should propagate in the testis no previous injury of the organ is necessary, save that caused by the injection, so that in this respect the testicle may be regarded as exceeding the skin surface in susceptibility to its presence. Possibly anatomical structure accounts for this difference; unless scarified the virus has no opportunity of adhering to and entering intimately into relation with the skin surface, while injection into the testicle brings the virus at once into immediate proximity to the tissues composing that organ.

The question which arises is whether the skin and testicular strain of virus remain identical in physiological properties. One has, of course, been derived from the other; but it remains to be considered whether in the course of adaptation the testicular strain has undergone modification.

Several criteria can be employed to decide this point: (a) type and course of the eruption of the skin of susceptible hosts (rabbit, calf, man); (b) characteristic lesion of the cornea of the rabbit; (c) histological processes within the lesions caused by the virus; (d) phenomena of immunity arising after vaccination; (e) resistance to the action of certain agencies, such as glycerin, ether, chloroform, phenol, drying, and heating.

It may be assumed that merely completely freeing vaccine virus from the usual bacteria which contaminate it in practice will not change its physiological properties. This probability is indicated by the effect of glycerination which reduces the bacterial content. The practical advantage of this reduction is obtained in milder vaccinal effects in man, which are equally protective with the severer effects resulting from bacterial coöperation.

Hence it may be hoped that the complete removal of the contaminating bacteria should still further improve the results of vaccination in that the secondary operation of bacteria contained within the virus will be entirely eliminated. The employment of such a pure strain virus will, at the same time, do away entirely with the potential danger of contamination of the virus with tetanus bacilli or spores, a subject which has always engaged the attention of producers, and respecting which special precautions are taken.

Skin Reactions.—Both skin and testicular strains of virus were inoculated simultaneously on different parts of the shaved skin surfaces of rabbits and calves, after which the course of the vaccination was followed. No differences were noted (figures 67, 68, and 69).

Cornea.—The rabbit's cornea inoculated with skin and testicular virus, respectively, undergoes the same series of changes, and in each case Guarnieri bodies are produced. The results are indistinguishable (figures 49 and 51).

Histological Lesions.—When the testicular strains of virus are reimplanted upon the skin of the rabbit and calf, and the minute histological changes produced are compared with those caused by the skin strains of virus, no essential differences have been detected (figures 47, 48, 50, and 52). Moreover, the microscopical changes in the testicles produced by skin or adapted testicular strains of virus are essentially identical.

Immunity Phenomena.

Considered from the practical standpoint the most important effect of vaccine virus is the immunity to reinoculation that it confers. Hence, experiments were conducted with the testicular strains in order to determine the immunity effects which it produces.

Experiment I.—Rabbit. First vaccination, Oct. 5, 1914, at three separate places on the shaved skin, with regular, glycerinated stock virus. Confluent vaccinal eruptions were produced.

Second vaccination, Feb. 12, 1915. The three places previously vaccinated were now revaccinated as follows: (a) an area with some of the virus used previously; (b) an area with rabbit testicular strain; (c) an area with bull testicular strain. The three areas reacted in identical manner: each area became congested, but neither vesicles nor papules appeared. The reddening of the skin was probably allergic in origin (20).

The control rabbit developed confluent eruptions from each of the three samples of virus.

Experiment II.—Rabbit. First vaccination, Oct. 5, 1914, at three separate places on the shaved skin, with a testicular strain of vaccine virus from a rabbit. Confluent vaccinal eruptions were produced.

Second vaccination, Feb. 12, 1915. The three places previously vaccinated were revaccinated as follows: (a) an area with some of the virus used previously; (b) an area with bull testicular strain; (c) an area with New York City Department of Health vaccine. On the following day slight transient scattered erythema was noted, but neither papules nor pustules.

The control rabbit developed typical confluent eruptions from each of the three samples of virus.

Experiment III.—Rabbit. First vaccination, Feb. 2, 1915, at three places on the shaved skin, with testicular strain from a bull calf (No. 4). Confluent vaccinal eruptions were produced.

Second vaccination, Feb. 17, 1915. The three places previously vaccinated were revaccinated as follows: (a) an area with some of the virus used in the first vaccination; (b) an area with a rabbit testicular strain; (c) an area with the regular New York City Department of Health vaccine. Only moderate allergic reactions were observed.

Two other rabbits similarly tested gave identical results with the above experiment, except for the presence in one of them of a slight erythema along the scarified lines, which lasted three days.

Controls on a normal rabbit showed that the strength of the vaccine strains employed for the foregoing experiments was such as to produce confluent eruptions.

The experiments described are conclusive in demonstrating that the skin and testicular strains of vaccine virus yield identical immunity reactions both as regards kind and degree.

*Testicular Cultivation as a Means of Propagation of Pure
Vaccine Virus.*

It is obvious that a method of propagating the vaccine virus free from bacterial contamination would remove all danger of accidental infection through the introduction at the time of vaccination of an impure preparation containing certain bacteria. Without stopping to consider how often severe effects have resulted from bacterial contamination of the virus it may be urged that the use of a pure preparation will altogether preclude the possibility of a mixed infection from this source. The indefinite propagation of a highly potent vaccine virus in the testicular tissues of animals is comparatively a simple procedure, so that by means of a strictly aseptic technique combined with careful bacteriological control of the product, it will now become a simple matter to supply the medical profession with an absolutely pure virus, for the purpose of vaccination. As is well known, the method hitherto practised for propagating vaccine consists in utilizing the emulsion of the skin pulp from vaccinated calves. The usual fresh pulp contains many and various bacteria, so that to free the pulp of them it must be left in contact for a period with 60 per cent. glycerin or 1 per cent. phenol, before it is regarded as ready for use on human subjects. Since the strength of the virus also deteriorates with time the process of vaccine preparation has not been perfected from the practical point of view. On the other hand, a pure virus should be ready for use as soon as the usual bacteriological and potency tests are completed, that is, within about one week after the preparation of the emulsion of the testicle containing it.

The introduction of a pure vaccine into the public health service would be welcome, even if it were somewhat more expensive to produce than the usual vaccine. As a matter of fact it appears to be even more economical than the latter, as the following calculation shows.

A male rabbit weighing about 3 kg. yields from 7 to 10 gm. of testicular material containing the pure culture of vaccine. When this amount of tissue is emulsified in from 15 to 20 c.c. of 50 per cent. sterile glycerin, it will yield from 20 to 28 c.c. of a finished product of high potency. A sample of this product is diluted 1 : 1,000 and tested on the skin of rabbits. If a confluent eruption results,

which may reasonably be expected, the stock suspension may be further diluted with four times its volume of sterile 50 per cent. glycerin, since a sample of glycerinated virus which produces a confluent eruption in a dilution of 1:200 is sufficiently strong for use in man. Hence a single rabbit may yield 125 c.c. of finished glycerinated virus, or even more.

The average yield from the calf, according to Fielder,¹⁰ is put at 40 to 100 gm. of fresh pulp, which emulsified with four parts of 50 per cent. glycerin gives 250 to 300 c.c. of vaccine virus ready for use within three months.

A comparison of the cost is in favor of the rabbit vaccine. For the preparation of 1,000 cubic centimeters of the finished vaccine product about 40 rabbits or 4 calves would be required. The initial cost of the former would be considerably smaller.

Moreover, the employment of rabbits instead of calves has advantages of another kind from those which have just been emphasized. The prevalence of foot-and-mouth disease among cattle has closed the market in many states to calves, so that the propagation of vaccine virus in the ordinary way has recently suffered. No such interruption is to be feared with respect to the use of rabbits. During the prevalence of such an epidemic it becomes essential also to keep the calves intended for vaccination under observation before use for a sufficient period to insure their freedom from foot-and-mouth disease infection.

Finally, it may be added that the bull may also come to be used for the testicular cultivation of the virus. However, the method has not yet been worked out for this animal as completely as it has for the rabbit. Hence it must be left to the future to determine whether the bull is strictly suitable for the purpose. This part of my work suffered interruption because of the prevailing epidemic of foot-and-mouth disease and the resulting quarantine upon cattle.

Standardization of Vaccine Virus.

Several methods are employed for determining the efficacy and potency of vaccine virus before distributing it for general use. The accepted methods agree in using animals before permitting the virus to be used on man. Theobald Smith recommends the skin of the calf, and requires that the eruptions produced there must correspond with those of the skin of a child. Calmette and Guérin (9, 21) and

¹⁰ On the other hand, Rosenau (1) gives the amount of fresh pulp from a calf as 20 to 40 gm., which will yield 50 to 120 c.c. of the glycerinated emulsion.

Camus (22) employ the skin of the rabbit; while Chaumier (23) and others¹¹ first employ animals, and then themselves observe the vaccination effects on a few children before issuing the virus. There is agreement among the authorities that the skin of the rabbit and calf are reliable indications of the effects produced on human subjects.

Henseval and Convent (24) consider that a preparation which produces on the skin of the rabbit almost confluent eruptions in a dilution of 1 to 500 should be considered a suitable vaccine. Such a sample always causes an uninterrupted eruption along a line (linear eruption) on the skin of a child. They recommend as still usable even a weaker specimen provided it produces a continuous eruption in a 1 to 100 dilution.

The testicular vaccine strains which the author prepared in rabbits either fulfill or exceed the above requirement, since a few samples produced confluent eruptions in dilutions of 1 to 10,000. On the other hand, the products from the testicles of the bull were less active, and a few only of the preparations reached the lower standard.

Pure Vaccination in Man.

The tests which were carried out in detail and with minute care in animals with the pure testicular strains of vaccine virus all indicated that the virus would produce typical vaccinal effects in the human subject.

Several adults, chiefly physicians familiar with the cultivation experiments, immediately volunteered for vaccination, which was carried out by the linear and scarification methods. Among the tests, which were all revaccinations, some were positive and others negative. In the positive results the vaccinal eruption was typical and no secondary infection arose to complicate or intensify the vaccination.

Among those in whom the vaccination was positive was a physician. After his experience he vaccinated his own child, three years and two months old, who had not previously been successfully

¹¹ The Department of Health of the City of New York issues only the preparations which have produced on primary vaccination cases fifteen typical takes on fifteen insertions made on five children, thus requiring 100 per cent. of positive takes for each sample of vaccine.

vaccinated. The linear eruption appeared on the fifth day, increased in intensity during the three succeeding days when it reached the maximum, and then receded. No pain or itching was complained of, and the highest temperature was 37.5° C., which was noted on the eighth day. On the fourteenth day scab formation was well advanced. I am indebted to the father of the child for the two photographs (figures 70 and 71) which illustrate the vaccinal effects on the fifth and sixth days.

SUMMARY.

Vaccine virus freed from all associated bacteria by means of suitable disinfecting agents can be propagated in a pure state in the testicles of rabbits and bulls. The virus cultivated in this manner is not only devoid of all bacteria, but appears capable of indefinite transfer from one animal to another. Sixty passages in rabbits of a pure strain have been made within one year.

Several transfers from testicle to testicle are required to bring about accurate adaptation of the virus to the testicular parenchyma, so that continued propagation in this way can be certainly secured. During the first transfers from testicle to testicle the activity of the virus may be less than the original skin specimen from which the pure strain was derived; but as the transfers proceed the activity rises until, when the adaptation is complete, the activity of the testicular equals that of the skin strain.

The multiplication of the virus within the testicle is maximum on the fourth or fifth day after inoculation; the quantity of virus remains about stationary until the eighth day, when diminution begins. At the expiration of five weeks no more virus could be detected in the testicle.

The vaccinal processes in the skin, cornea, and testicle of rabbits are practically identical whether the virus employed for the inoculation has been the original skin strain or the pure testicular strain; and the skin lesions produced in the calf with the two strains are also identical.

In conformity with the finding mentioned in the last paragraph it has been found that human beings react to the pure testicular strain of vaccine virus in an entirely typical manner. In the case

both of original vaccination and revaccination the vaccinal effects cannot be distinguished from those arising from uncomplicated skin virus.

Pure strains of testicular virus are readily produced, and once secured they may be propagated in a pure state by the method described in rabbits or bulls without difficulty and with economy. The pure strains thus obtained should supply an ideal form of virus for employment in the vaccination of human beings.

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EXPLANATION OF PLATES.

PLATE 39.

FIG. 1. The right testicle shows swelling due to the injection of pure vaccine virus made five days previously; the left side is normal.

FIGS. 2 to 12. The conditions of the testicles at various stages in the vaccinal processes produced by means of a pure vaccine virus, as follows: 2, 48 hours; 3, 3 days; 4, 4 days; 5, 5 days; 6, 6 days; 7, 7 days; 8, 8 days; 9, 9 days; 10, 10 days; 11, 11 days; and 12, 12 days after the inoculation. The yellowish grey mottles of irregular dimension can be seen in the specimens shown in figures 4, 5, and 6. The marked atrophy of the organ is easily noticeable in the specimens shown in figures 9, 11, and 12. All natural size.

PLATE 40.

FIG. 13. A testicle four days after inoculation with pure vaccine virus. It shows marked vascular injection, edema, and several small yellowish grey spots visible on the surface.

FIG. 14. The organ on section, in which several lobules can be seen undergoing necrosis, as indicated by the greyish yellow, somewhat indurated areas. The parenchyma has bulged out of the tunica albuginea on account of the edema.

FIGS. 15 and 16. Two specimens of testicles at the height of the vaccinal processes, one (figure 16) with hemorrhagic reactions. In the latter instance the necrotic changes are much more pronounced, as is indicated by the general yellowish grey color of the lobules on section.

PLATES 41 AND 42.

FIG. 17. The structure of the testicle of a normal rabbit under a low power (magnified 13 diameters).

FIG. 18. Diffuse interstitial infiltration of the vaccinated testis after three days. The compression of the tubules through intertubular edema and infiltration is well shown.

FIG. 19. A four day specimen which presents a large, irregularly defined infiltration and the destruction of the tubules.

FIGS. 20 to 24. Gradual absorption of the inflammatory products with distinct diminution of tubules. There are still many foci of infiltration.

FIGS. 25 and 26. The structure of testicles which had been vaccinated three and seven weeks previously.

FIG. 27. A longitudinal section of a testicle which shrank as the result of vaccination fifty-six days previously. There are only a few tubules left, all without any spermatogenetic activities. Magnification: $\times 13$ throughout.

PLATE 43.

FIG. 28. A section of normal rabbit's testicle stained by the Borrel-Calkin method (25).

FIGS. 29 and 29 a. The peculiar round or oval bodies of varying size within the affected cellular elements in the vaccinated testicles of rabbits. These bodies appear brilliantly red when stained by the Borrel-Calkin method, and they resemble the vaccine bodies so called. These bodies may have been derived from the nuclei of the cells under the influence of the vaccine virus. Magnification: $\times 340$ throughout.

PLATE 44.

FIGS. 30 and 31. The structure of a normal testicle of the rabbit.

FIG. 32. An early polynuclear infiltration of the interstitial spaces of a vaccinated testicle of the rabbit after twenty-four hours.

FIG. 33. A similar but more advanced stage after three days.

FIG. 34. Extensive involvement of the testicular tubules as well as the interstitial tissues after four days.

FIG. 35. A focus where polynuclears are seen to have begun to break up after five days.

FIG. 36. An area of extensive necrosis occurring in a specimen removed after four days.

FIG. 37. The cellular infiltration and fibrin in a specimen after five days (Weigert stain).

FIG. 38. An intratubular infiltration occurring in a specimen seven days after inoculation. The cells are basophilic leucocytes. Magnification: $\times 170$ throughout.

PLATE 45.

FIG. 39. A tubule completely filled with leucocytes in a specimen eight days after inoculation.

FIGS. 40 and 41. Enormous cellular infiltration along the tunica albuginea in nine day specimens.

FIG. 42. The detachment of the degenerated testicular cells from the wall of affected tubules in a twelve day specimen.

FIG. 43. A focus of infiltration in a twenty-one day specimen. Such a focus is rather rare in old specimens.

FIG. 44. A focus of infiltration which was found in a fifty-six day specimen.

FIG. 45. The general changes of the structure of the vaccinated testicle after fifty-six days.

FIG. 46. The infiltration of the tunica vaginalis in a rabbit with polynuclear leucocytes in a four day specimen.

FIGS. 47 and 48. The vaccinal reactions on the skin of rabbits. Magnification: $\times 170$ throughout.

PLATE 46.

FIG. 49. The Guarnieri, or vaccine bodies, so called, in the corneal epithelium of a rabbit inoculated with pure testicular vaccine virus. Three day specimen.

FIG. 50. The vaccinal reaction in the skin of a rabbit inoculated with the testicular strain of vaccine virus. Stained with Calkin's modification of Borrel's method.

FIG. 51. The vaccine bodies in a rabbit's cornea inoculated with regular vaccine strain.

FIG. 52. Normal rabbit skin stained with the Borrel-Calkin method.

PLATE 47.

FIG. 53. The inoculated (left) and normal (right) testicles of bull 2 on the sixth day after the injection of pure testicular vaccine from a rabbit. The left side is seen to be somewhat larger than the right. The skin shows typical vaccinal pustules also produced with the same material.

FIG. 54. The inoculated testicle and the tunica vaginalis of the same animal, the latter showing much hemorrhage.

FIG. 55. The same organ on section. One notices the hemorrhagic foci and necrotic area.

FIGS. 56 and 57. The testicles of bull 6. The larger one was inoculated with the testicular, and the smaller with a skin strain of vaccine virus, and both were removed on the sixth day.

PLATE 48.

FIG. 58. The structure of a normal testicle of a young bull. One recognizes at once that the spermatogenesis is in full display.

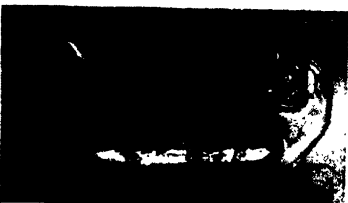
FIGS. 59 to 61. The reactions following the injection of pure testicular vaccine strain into the organ. They represent changes found on the sixth day.

FIGS. 62 to 66. The vaccinal processes in the testicles of bull calves. The changes are similar to those found with young bulls and rabbits, except that the animals were too young to have any spermatogenesis, even in the unaffected areas of the organ. Both in the young bulls and in the bull calves the interstitial infiltration with polynuclear leucocytes is marked. Magnification: $\times 170$ throughout.

PLATE 49.

FIG. 67. The typical vaccinal effect on the skin of a rabbit inoculated with a pure testicular strain of vaccine virus on the sixth day.

FIG. 68. A confluent eruption on the skin of a rabbit vaccinated with a pure testicular strain.





13



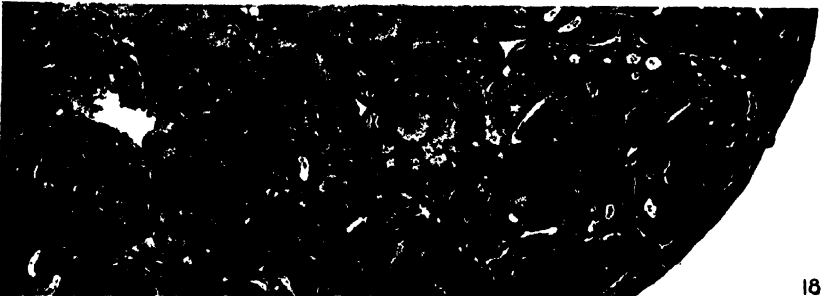
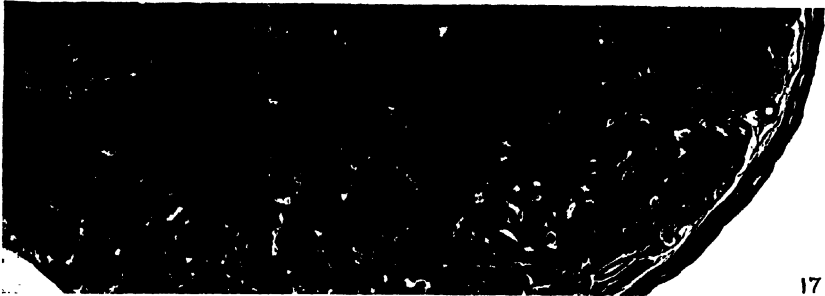
14

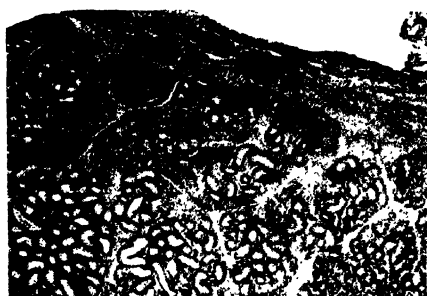


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16





23



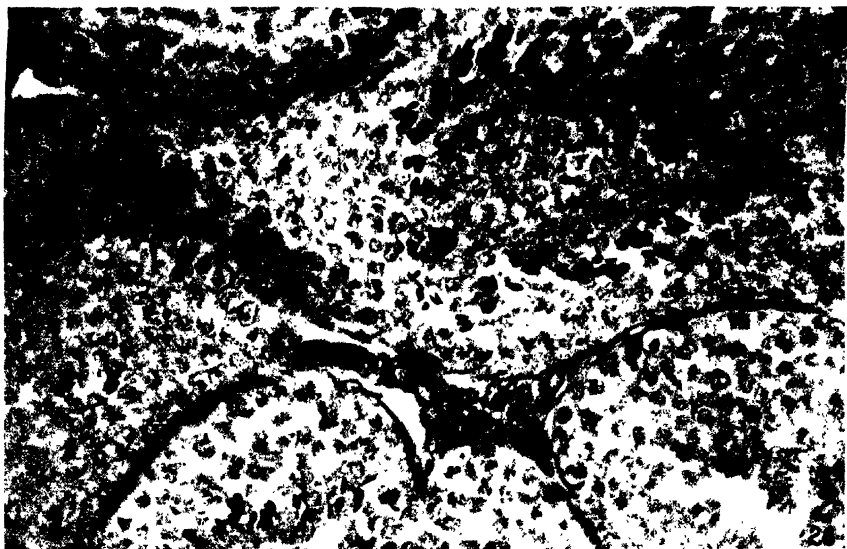
24

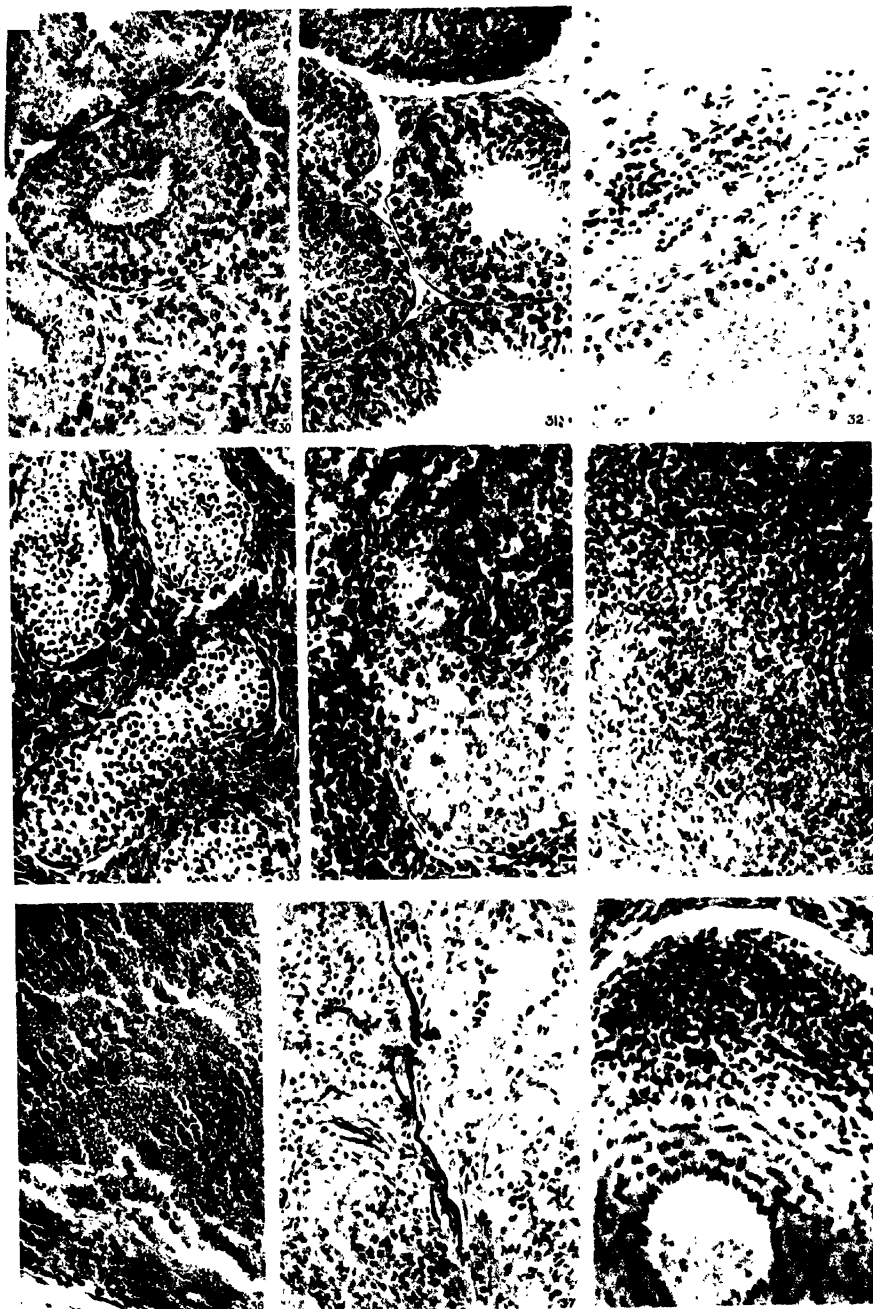


25

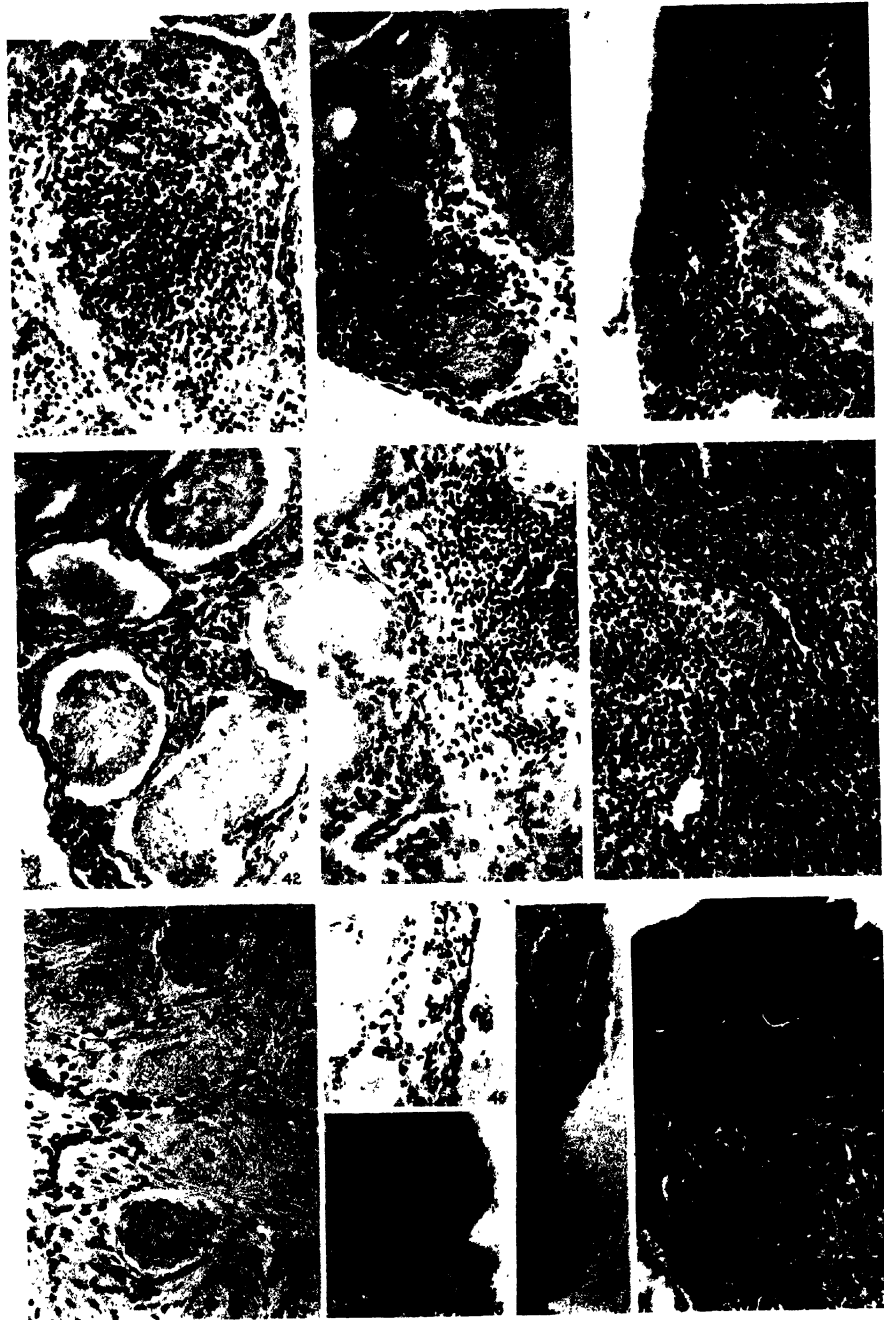


27





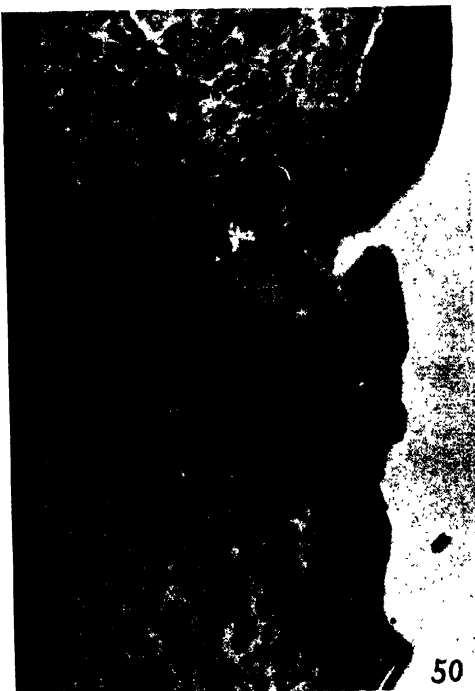
(Noguchi: Cultivation of Vaccine Virus.)



(Noguchi: Cultivation of Vaccine Virus.)



49



50

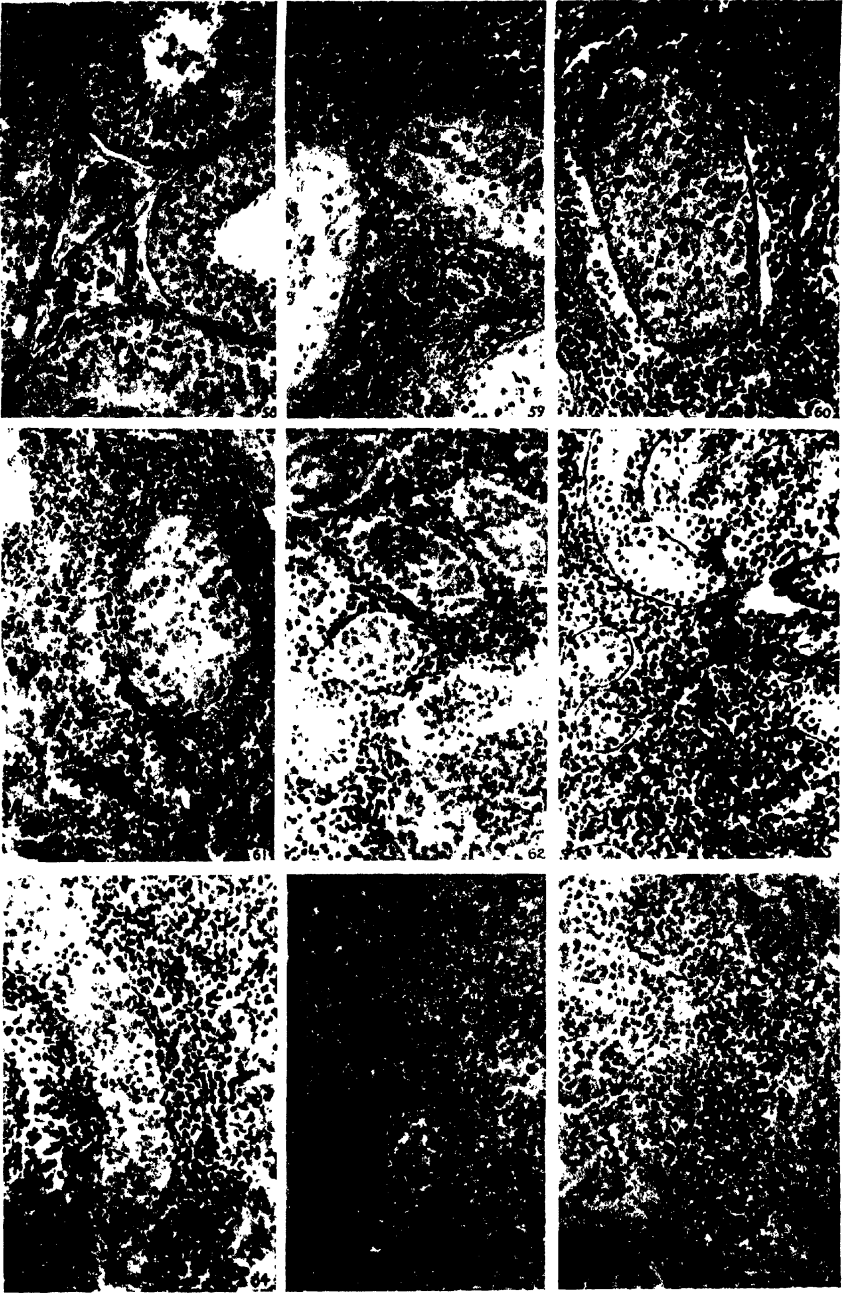


52

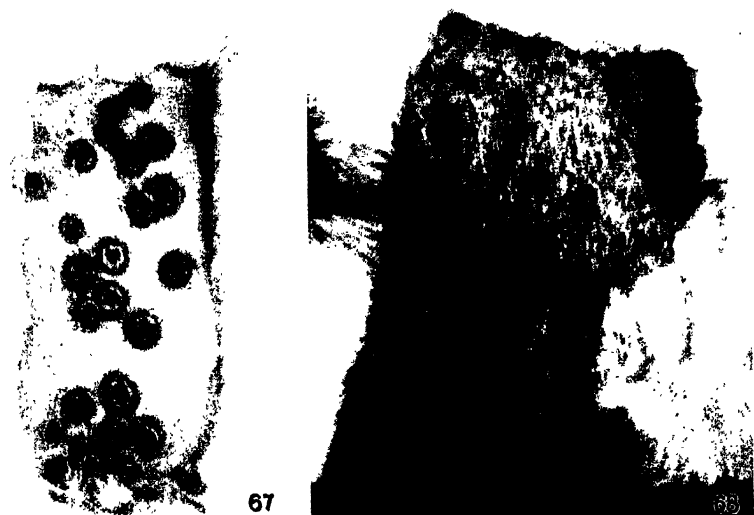
(Noguchi: Cultivation of Vaccine Virus.)



(Noguchi: Cultivation of Vaccine Virus.)



(Noguchi: Cultivation of Vaccinia Virus.)



(Noguchi: Cultivation of Vaccine Virus.)

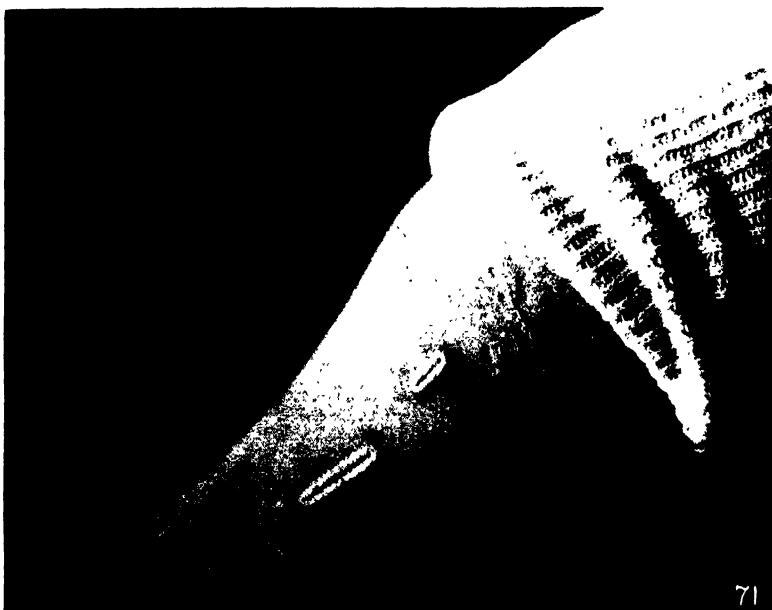


FIG. 69. The typical vaccinal effect on the skin of a calf inoculated with a pure testicular strain of vaccine virus obtained from a rabbit, on the sixth day. Natural size.

PLATE 50.

FIGS. 70 and 71. The fifth and the sixth day phases of the first vaccination in a child three years and two months old. The virus was prepared in the rabbit according to the method described in this work, and was absolutely free from bacteria. Notice the uninterrupted linear eruption on both sides. In figure 71 the mottled appearance around the site of vaccination is due to the adhesion of the vaccine shield.

ADULT TERTIAN MALARIAL PARASITES ATTACHED TO PERIPHERAL CORPUSCULAR MOUNDS. THE EXTRACELLULAR RELATION OF THE PARASITES TO THE RED CORPUSCLES.*

By MARY R. LAWSON, M.D.

(From the Laboratory of Dr. Mary Rowley-Lawson, New London.)

PLATES 55 TO 58.

Received for publication, March 29, 1915.

Many of the misconceptions in regard to the morphology and biology of the malarial parasite have risen, I believe, from the fact that the majority of observers have believed the parasite to be intracellular or submerged beneath the surface of the red corpuscle, and that each parasite grew up and completed its life cycle within a single red corpuscle, the segmentation of the parasite corresponding with the final destruction of the red corpuscle.

I have shown in previous publications¹ that the malarial parasite in the course of its development destroys several red corpuscles, migrating from one to another, and that when it is not free in the blood serum, it is attached to the outer surface of the red corpuscle. As Laveran² describes it, the parasites are "now free in the blood, now adherent to blood corpuscles."

The Method by Which the Malarial Parasite Secures Attachment to the Surface of the Red Corpuscle.—The parasite attaches itself to the outer surface of the red corpuscle by means of pseudopodia encircling a portion of the hemoglobin substance which has been squeezed up by the parasite into a mound with a circular base and a more or less rounded apex. The pseudopodia encircle the mound

* Aided by a grant from The Rockefeller Institute for Medical Research.

¹ Rowley-Lawson, M., *Arch. Int. Med.*, 1912, ix, 420; *Jour. Exper. Med.*, 1913, xvii, 324; 1914, xix, 450, 523.

² Laveran, A., *Paludism*, translated by Martin, J. W., London, 1893, 12.

at its base. The corpuscular mounds may be encircled by a single pseudopodium or by two pseudopodia meeting and fusing.

I believe that the first pseudopodia thrown out arise from the cytoplasm of the parasite near the nucleus. Other (accessory) pseudopodia may be thrown out from any part of the cytoplasm, whenever it becomes necessary for the parasite to secure a firmer hold on the red corpuscle; *i. e.*, when a corpuscular mound to which it is attached becomes dehemoglobinized (decolorized), or as the parasite increases in size.

A very young parasite may find it necessary to use its entire cytoplasm in order to encircle a corpuscular mound, thus giving the appearance, when attached to a surface mound, of a very slender ring with a chromatin dot. When a corpuscular mound encircled by a young parasite is viewed from above, the characteristic ring-form parasite picture is seen.

Young Parasites Attached to Peripheral Corpuscular Mounds.—(Figures 1 to 10.) The above interpretation of the ring-form parasite is confirmed by seeing the mounds of corpuscular substance projecting at the periphery of the red corpuscles encircled by the pseudopodia of the parasites.

The encircling pseudopodia may be wholly or in part obscured by the nuclei of the attached parasites; but frequently they may be traced around the peripheral mounds (figures 1 and 10).

Peripheral corpuscular mounds encircled by young parasites are best seen when the corpuscle is newly invaded and the hemoglobin of the mound entirely or nearly intact. As a rule, the corpuscular mounds are rapidly decolorized, proceeding as the parasite digests the hemoglobin. The various stages between the unaltered deeply colored mounds and those which are entirely decolorized may be observed (figures 1 to 10). Rarely the red corpuscle is decolorized before the corpuscular mound.

The decolorized corpuscular mound has been called the "achromatic area" and "milky zone," and many observers have believed it to be an essential part of the parasite.

As additional proof of the extracellular relation of the young parasites to the red corpuscle, the nuclei or the protoplasm may often be seen to project beyond the periphery of the red corpuscle (figures 6, 7, 9, and 10).

Pigment in Connection with Young Parasites.—The secretion of pigment by the young tertian parasites seems to follow no known rule. Its first appearance, even in the parasites of a single blood smear, is variable. The decolorization of a corpuscular mound (or even of an entire red corpuscle) may be complete without the appearance of any demonstrable pigment in the young parasite attached to it (figure 6, see parasite at o), and a young parasite without pigment may be of larger growth than a parasite with pigment. And one may see two parasites apparently of equal growth, one with and one without pigment (compare parasites without pigment in figures 1, 3, and 5, with the parasite showing a pigment granule at o in figure 2). And in instances of multiple infection of corpuscular mounds by two young parasites of an apparently equal growth, only one of these parasites may show pigment (figure 7).

This irregularity as to the time when the first pigment appears in the individual young parasite may have no significance other than that certain parasites are slower than others in securing attachment to the red corpuscles, and it may be that some of the parasites are less active than others in the secretion of pigment.

While the presence of pigment in the young parasite has a positive value, its absence does not mean that the parasite has not done its share of injury to the red corpuscle.

Infection of Corpuscular Mounds by More than One Young Parasite.—Infection of corpuscular mounds by two or more parasites may be seen (figures 4, 5, x, 6, x, and 7). It has no significance other than accidental occupation. Parasites occupying the one corpuscular mound are usually of one brood and, therefore, in the same stage of development; but parasites of varying ages may also be seen occupying the one corpuscular mound (figure 6, x).

In multiple infections of corpuscular mounds the parasites proceed with the destruction of the mounds in the same way as do single parasites.

Adult Tertian Parasites Attached to Peripheral Corpuscular Mounds.—(Figures 11 to 72, and 75 to 108.) While several observers have conceded that the young parasite may be extracellular for a certain time, there has been, with but few exceptions, quite a unanimity of opinion amongst the older writers that the adult para-

site is always intracellular. I have been asked several times if it was possible to demonstrate any appreciable number of adult tertian parasites attached to peripheral corpuscular mounds. These inquiries led to an exhaustive study in search of the same.

Adult parasites are usually seen attached to surface mounds; but peripheral mounds may be overlooked or not recognized, except by the expert observer. Here and there you find adult tertian parasites attached to perfectly recognizable peripheral corpuscular mounds. I believe that adult tertian parasites attached to peripheral corpuscular mounds are only to be found in appreciable numbers in any one smear, soon after a parasitic migration when the infected red corpuscles are not badly damaged; then the peripheral mounds may show more or less of hemoglobin content. But such a condition is so transient that you may have to search for days and examine many specimens before you find what you want.

Corpuscular mounds are quickly dehemoglobinized by adult parasites (figures 50 at 0, 65, 69 to 73, and 105 to 108); I believe more quickly than by the young parasites.

If the tertian malarial parasites were attached to but one red corpuscle during their life time and did not migrate to fresh red corpuscles, I believe that it would be practically impossible to find peripheral corpuscular mounds in connection with the adult parasites.

The adult tertian parasites follow the same procedure in attaching themselves to the red corpuscles as do the young parasites. Primary and accessory pseudopodia encircle surface mounds. As a rule, the pseudopodia which encircle the peripheral corpuscular mounds arise from the cytoplasm of the parasites in the vicinity of the nuclei, as is the case with the young parasites.

The bases of some of the corpuscular mounds in connection with the adult parasites are more constricted than is usual with the mounds seen in connection with the young tertian parasites; *i. e.*, figures 12, 20, 21, and 23. This would indicate greater strength exerted by the adult parasites when encircling the corpuscular mounds.

The nuclei of the adult parasites usually hide from view the encircling pseudopodia, but occasionally the pseudopodia may be seen en-

circling the peripheral mounds (figures 11, 12, 13, 14, 16, 27, 32, 39, 52, 56, 57, 59, 61, 69, 73, 75, 76, 77, 79, 96, 97, 101, 102, and 106).

The nuclei of the adult parasite often extend beyond the periphery of the red corpuscle (figures 12 and 14, 76 and 79), which would not be the case were the parasite intracellular. And some of the adult parasites are so attached to the surface of the red corpuscle that even to the untrained eye they admit of no other interpretation than that they are attached to the outside of the red corpuscle (figures 74 and 109).

Pigment in Connection with Adult Parasites.—Adult parasites, apparently in similar stages of development, may show not only a variation in the amount of pigment secreted, but a variation in the size and shape of the granules. Various explanations have been given to account for this, but I shall not enter into a discussion of them in this paper.

Multiple Infection of Corpuscular Mounds by Adult Parasites.—More than one adult parasite may be seen to be attached to a single corpuscular mound (figures 18, 19, 44, 63, 72, 86, 87, and 100). As is the case with the young parasites, adults occupying the one mound may be in similar or varying stages of development. Their occupancy of the one mound I believe to be accidental.

I have never found any evidence of the conjugation of malarial parasites. Two parasites attached to one surface mound, with their pseudopodia encircling it superimposed, when viewed from above might give an appearance of conjugating parasites, provided one believed the parasites to be intracellular; but when one knows that they are attached to the one corpuscular mound on the surface of the red corpuscle, then their position in relation to each other is easily explained.

Mounds of hemoglobin substance projecting out at the periphery of the red corpuscles, encircled by the pseudopodia of the two adult parasites, assist in the interpretation of the surface mounds encircled by two adult parasites.

The infected red corpuscles in the pictures which I have selected to illustrate this article show no evidence of crenation or other technical injury.

SUMMARY.

1. The malarial parasite is extracellular throughout its entire life cycle; that is, when it is not free in the blood serum, it is attached to the external surface of the red corpuscle.

2. Adult parasites follow the same procedure in attaching themselves to the outer surface of the red corpuscles as do the young parasites.

3. Adult parasites are most frequently seen attached to surface corpuscular mounds.

4. Corpuscular mounds projecting at the periphery of the red corpuscles and encircled by the pseudopodia of adult parasites, are proof positive of the extracellular relation of the adult parasite to the red corpuscle.

5. Adult parasites attached to peripheral corpuscular mounds are only found in appreciable numbers when the red corpuscles are not badly damaged, so that the mounds show more or less hemoglobin content.

6. The nuclei or protoplasm of adult parasites extending beyond the periphery of the red corpuscles is additional evidence of the extracellular relation of the parasites to the red corpuscle.

EXPLANATION OF PLATES.

PLATE 55.

Tertian Malarial Parasites Attached to Peripheral Corpuscular Mounds.

Magnification, $\times 1,900$.

FIG. 1. A young parasite attached to a peripheral corpuscular mound. The pseudopodium may be seen encircling the mound.

FIG. 2. A young parasite attached to a peripheral corpuscular mound. A pigment granule may be seen at o.

FIG. 3. A young parasite attached to a partly decolorized corpuscular mound.

FIG. 4. Two young parasites attached to one peripheral corpuscular mound. The nucleus of one parasite partly overlies that of the other parasite, giving the appearance of one large nucleus, but a careful examination will show the presence of two parasites.

FIG. 5. A young parasite attached to a peripheral corpuscular mound and two young parasites attached to a single corpuscular mound on the surface of the red corpuscle.

FIG. 6. Two young parasites, varying somewhat in size, attached to a peripheral corpuscular mound; and at o, a young parasite attached to a completely decolorized peripheral mound.

FIG. 7. Two tiny parasites (it requires the entire cytoplasm of each parasite to encircle the mound) attached to a decolorized peripheral mound. One of the parasites shows a granule of pigment at x.

FIG. 8. A young parasite attached to a peripheral mound which is almost decolorized. A granule of pigment may be seen in connection with the parasite.

FIG. 9. A young parasite attached to a decolorized peripheral mound.

FIG. 10. A young parasite attached to an almost decolorized peripheral mound. A careful examination will show the encircling pseudopodium on the under surface of the mound.

FIG. 11. Two parasites attached to peripheral corpuscular mounds; one of the parasites is much smaller than the other.

FIGS. 12, 13, and 14. Adult parasites attached to peripheral corpuscular mounds. The pseudopodium of each parasite may be seen encircling the mound.

FIGS. 15, 16, and 17. Adult parasites attached to peripheral corpuscular mounds. Figure 15 shows the nucleus of the parasite overlying the encircling pseudopodium. In figure 16 the pseudopodium may be seen on the under surface of the mound. In figure 17 the nucleus of the parasite partly overlies the encircling pseudopodium.

FIGS. 18 and 19. Two adult parasites attached to the one peripheral corpuscular mound.

FIG. 20. An adult parasite attached to a peripheral mound. The mound is somewhat constricted at the base.

FIG. 21. An adult parasite attached to what is really a surface mound, with the periphery of the corpuscle so turned over that the surface mound presents as a peripheral mound. This mound is somewhat constricted at the base.

FIGS. 22, 23, 24, 25, and 26. Adult parasites attached to peripheral corpuscular mounds. Figure 23 shows two peripheral mounds, one of them being very much constricted at its base.

PLATE 56.

Adult Tertian Malarial Parasites Attached to Peripheral Corpuscular Mounds.

Magnification, $\times 1,900$.

FIGS. 27 to 50. Adult parasites attached to peripheral mounds. Figures 29 and 33 show each parasite attached to two peripheral mounds. Figure 50 shows an entirely decolorized mound at o. Figure 44 shows two parasites attached to one peripheral mound.

PLATE 57.

Adult Tertian Malarial Parasites Attached to the External Surface of the Red Corpuscles.

Magnification, $\times 1,900$.

FIGS. 51 to 59. Adult parasites attached to peripheral mounds.

FIG. 60. An adult parasite attached to what is really a surface mound. The periphery of the corpuscle is turned over so that the surface mound presents as a peripheral mound.

FIGS. 61 and 62. Adult parasites attached to partially decolorized peripheral mounds.

FIG. 63. Three parasites attached to one peripheral mound. The mound is much decolorized.

FIG. 64. An adult parasite attached to a partially decolorized peripheral mound.

FIG. 65. An adult parasite attached to a decolorized corpuscular mound.

FIG. 66. An adult parasite attached to a peripheral mound. The mound shows a partial decolorization.

FIGS. 67 and 68. Adult parasites attached to peripheral corpuscular mounds which have been partly dehemoglobinized by the action of the parasites.

FIGS. 69, 70, and 71. Adult parasites attached to completely decolorized corpuscular mounds.

FIG. 72. Two adult parasites attached to a completely decolorized corpuscular mound.

FIG. 73. An adult parasite attached to a decolorized corpuscular mound.

FIG. 74. An adult parasite attached to the surface of the red corpuscle in such a manner that even the most skeptical observer would not think it to be other than extracellular.

PLATE 58.

Colored Photographs of the Parasites Shown in Plates 55, 56, and 57.

Magnification, $\times 1,900$.

FIG. 75. (Corresponds to figure 11.) Two parasites in varying stages of development attached to peripheral corpuscular mounds.

FIGS. 76, 77, 78, and 79. (Correspond to figures 12, 13, 28, and 14.) Adult parasites attached to peripheral corpuscular mounds. The pseudopodia of the parasites in figures 76, 77, and 79 may be seen encircling the mounds.

FIG. 80. (Corresponds to figure 15.) Adult parasite attached to a peripheral mound. The nucleus of the parasite overlies the encircling pseudopodium.

FIG. 81. (Corresponds to figure 16.) An adult parasite attached to a peripheral mound. The pseudopodium may be seen encircling the mound.

FIGS. 82, 83, and 84. (Correspond to figures 17, 20, and 22.) Adult parasites attached to peripheral mounds. Figure 83 shows the base of the mound to which the parasite is attached to be slightly constricted.

FIG. 85. (Corresponds to figure 21.) An adult parasite attached to what is really a surface mound. The periphery of the red corpuscle is turned over so that the surface mound presents as a peripheral mound.

FIGS. 86 and 87. (Correspond to figures 18 and 19.) Two adult parasites attached to one corpuscular mound.

FIGS. 88, 89, and 90. (Correspond to figures 24, 25, and 47.) Adult parasites attached to peripheral corpuscular mounds.

FIG. 91. (Corresponds to figure 29.) An adult parasite attached to two peripheral corpuscular mounds.

FIGS. 92, 93, and 94. (Correspond to figures 31, 34, and 35.) Adult parasites attached to peripheral corpuscular mounds.

FIG. 95. (Corresponds to figure 23.) An adult parasite attached to two peripheral corpuscular mounds. One of the mounds is very much constricted at its base.

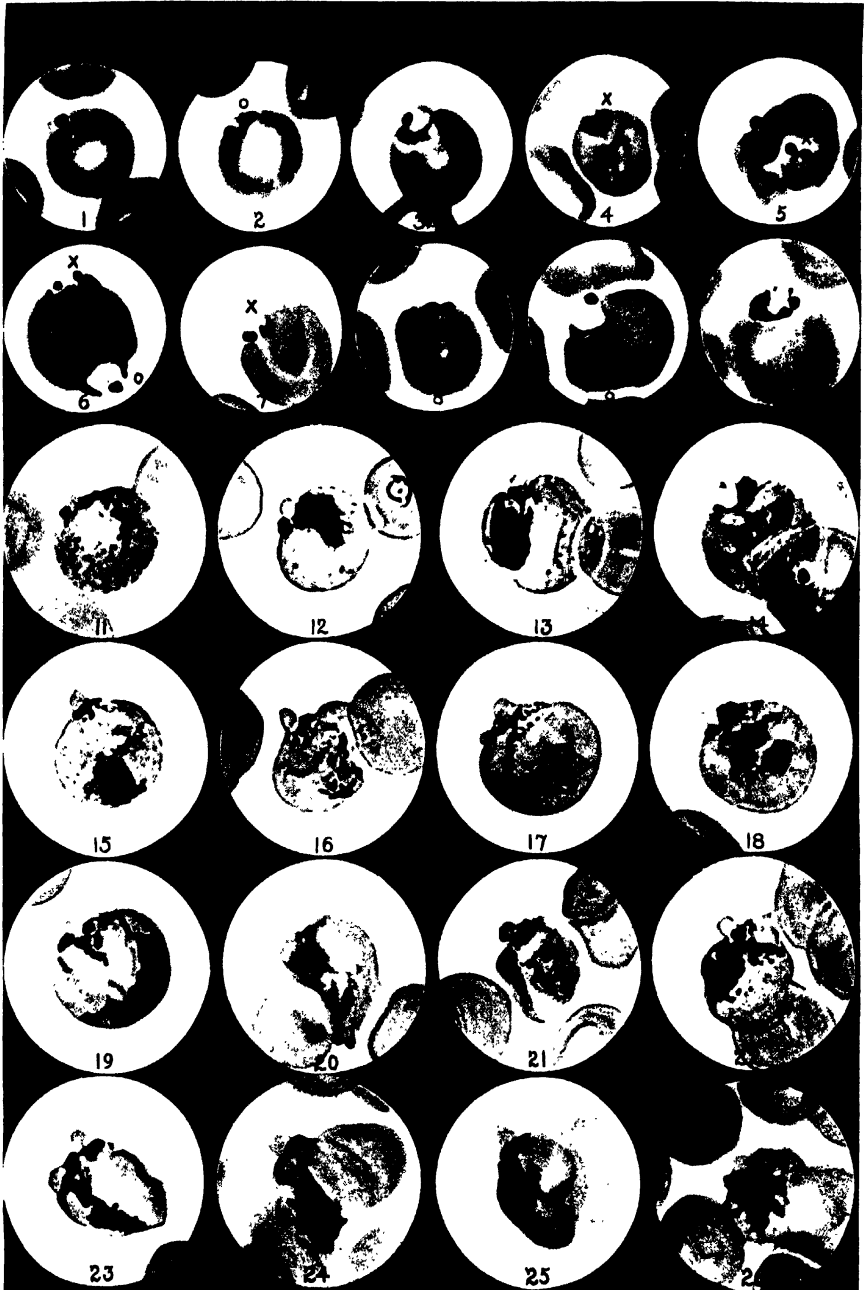
FIGS. 96, 97, 98, and 99. (Correspond to figures 39, 52, 41, and 42.) Adult parasites attached to peripheral corpuscular mounds.

FIG. 100. (Corresponds to figure 63.) Three parasites attached to one corpuscular mound.

FIGS. 101, 102, 103, and 104. (Correspond to figures 37, 61, 62, and 40.) Adult parasites attached to more or less decolorized corpuscular mounds.

FIGS. 105, 106, 107, and 108. (Correspond to figures 50, 69, 70, and 71.) Adult parasites attached to decolorized corpuscular mounds.

FIG. 109. (Corresponds to figure 74.) An adult parasite attached to the external surface of the red corpuscle.



(Lawson: Adult Tertian Malaria Parasites)



(Lawson: Adult Tertian Malarial Parasites.)



(Lawson: Adult Tertian Malarial Parasites.)



A. J. H. & C. J. H. H. H.

(Lawson: Adult Tertian Malarial Parasites.)

A STUDY OF THE EFFECT OF SENSITIZATION ON THE DEVELOPMENT OF THE LESIONS OF EXPERIMENTAL PNEUMONIA IN THE RABBIT.*

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Received for publication, November 5, 1914

The analogies between the phenomenon of anaphylaxis in the laboratory experiment and the development and course of spontaneous pneumonia in man are, perhaps, more striking than those shown by any other infection. In pneumonia, moreover, conditions of hypersusceptibility might conceivably be one of the factors or the dominant factor, not only in the systemic febrile reaction but also in the localized and unique lung reaction such as is found only in man. The following study records a series of experiments in which an attempt was made to ascertain whether any definite and constant relationship between conditions of hypersusceptibility and the development of pneumococcus lesions of the lobar type in animals could be established.

Wadsworth,¹ in 1904,—before the phenomenon of anaphylaxis had been associated with infections,—stated that typical lobar lesions developed in a considerable number of partially immunized rabbits after tracheal injection of small quantities of pneumococcus culture. These lesions, absent when normal rabbits had been injected, were also much less extensive or lacking when highly immunized animals were used. Viewed from our present knowledge of anaphylaxis the acute lung reaction might possibly have been due to a state of hypersusceptibility unintentionally caused while attempting to induce a slight immunity as a predisposing condition to the development of the lobar type of lesion.

Typical lobar pneumonia has been successfully incited in the dog by Lamar and Meltzer² and others by intrabronchial insufflation of large quantities of pneumococcus culture. In rabbits the reaction has been induced experimentally with difficulty, as, owing to their extreme susceptibility to pneumococcus infection, a general bacteremia without characteristic lung involvement is usual.

* Aided by a grant from The Rockefeller Institute for Medical Research.

¹ Wadsworth, A., *Am. Jour. Med. Sc.*, 1904, cxxvii, 851.

² Lamar, R. V., and Meltzer, S. J., *Proc. Soc. Exper. Biol. and Med.*, 1909-10, vii, 102; *Jour. Exper. Med.*, 1912, xv, 133.

Rasquin³ stated that tracheal injection of pneumococci gave typical pneumonias in only 7 to 8 per cent. of his rabbits, but when serum from a dog immunized to rabbit serum was added to the culture, 96 per cent. developed typical catarrhal lesions. Winternitz and Hirschfelder,⁴ however, by tracheal insufflation of large amounts—4 to 5 c.c.—of concentrated culture were able to obtain extensive fibrinous involvement in a large proportion of fairly small rabbits.

Friedberger⁵ and later Schlecht and Schwenker⁶ reported a cellular bronchopneumonia produced by spraying horse serum into the tracheas of sensitized guinea pigs. Anaphylactic shock and pneumonic changes in the lungs of sensitized guinea pigs following intratracheal injection of small amounts of horse serum also have been reported by Ishioka.⁷

While the rabbit has proved more resistant to sensitization than the guinea pig, and the results generally less constant and less marked, definite anaphylactic reactions with bacterial proteins have been obtained. Neufeld and Dold⁸ and Rosenow⁹ incited acute toxic symptoms in the rabbit similar to those of anaphylaxis by using a pneumococcus culture and normal or immune serum-complement mixture, such as Friedberger had obtained with other bacteria. The toxic split products of pneumococci obtained by Vaughan, the autolysates of Rosenow, and the lytic bile extracts of Cole have all caused acute toxic reactions in the rabbit. Apparently in none of the experiments with bacteria has the toxic dose been given tracheally, nor has the local reaction called forth by it been studied.

In the present study two hypotheses have been kept in mind: one which would be strengthened by negative results, that in lobar pneumonia, notwithstanding its extraordinarily rapid and exceptional course, the progress is still a definitely progressive one and not dependent on conditions such as give rise to the sudden and eruptive phenomena of anaphylaxis; the second, suggested by the abrupt onset and sudden termination of the disease, by certain local reactions, and by the not infrequent clinical history of previous infection suggestive of sensitization, that its unique and striking features are of an anaphylactic nature. In the latter case pneumonia may be considered either as mainly a bacteremic or toxemic condition, the lung lesions being a local and comparatively unimportant feature of the disease as a whole, and the anaphylactic reaction a general and systemic phenomenon. Or, the lungs with their system of tubes

³ Rasquin, E., *Arch. de méd. expér. et d'anat. path.*, 1910, xxii, 804.

⁴ Winternitz, M. C., and Hirschfelder, A. D., *Jour. Exper. Med.*, 1913, xvii, 657.

⁵ Friedberger, E., *Deutsch. med. Wchnschr.*, 1911, xxxvii, 481.

⁶ Schlecht, H., and Schwenker, G., *Deutsch. Arch. f. klin. Med.*, 1912, cviii, 405.

⁷ Ishioka, S., *Deutsch. Arch. f. klin. Med.*, 1912, cvii, 500.

⁸ Neufeld, F., and Dold, H., *Berl. klin. Wchnschr.*, 1911, xlviii, 55.

⁹ Rosenow, E. C., *Jour. Infect. Dis.*, 1911, ix, 190.

and air spaces may provide a particularly favorable environment and may act as a coöperating test-tube in which the cell complex furnishes the antibody, and the invading pneumococci the toxic agent. The resulting toxic substances, acting locally, might then incite exudative reactions in the lung, and on being absorbed give rise to the characteristic febrile reaction, the bacteremia being a secondary and transitory phenomenon.

Methods.—The pneumococcus organisms used were A, a virulent strain (the Neufeld culture), obtained through the kindness of Dr. Cole of the Hospital of The Rockefeller Institute; B, one of moderate virulence, but which had given evidence of unusual toxicity; and AA, an avirulent culture of A, attenuated by long continued growth on agar. Derivatives of eighteen to twenty-four hour meat infusion broth cultures were used for sensitization. The culture was centrifugalized and the supernatant fluid passed through a Berkefeld and then a Pasteur filter. The sediment was washed twice, suspended in salt solution equal to one-half or one-quarter of the original culture, and heated for thirty minutes at 52° to 55° C. Usually 0.1, 0.5, 7.5, and 15 cubic centimeters of filtrate or dead cell suspension were given intravenously to groups of rabbits. Two weeks later one cubic centimeter of eighteen to twenty-four hour live broth cultures was given tracheally to the rabbits. The same or a larger volume of concentrated suspension of living pneumococci cultivated aerobically in broth, or anaerobically under oil, or in large Petri dishes, was later used in a second series of rabbits. The effect of repeated sensitizing or toxic doses was also tested.

In studying the reactions incited by tracheal injection of serum and culture, mixtures were made of one cubic centimeter of live broth culture, and 0.1 or 0.5 of a cubic centimeter of sera from normal rabbits, or from animals immunized with pneumococcus filtrates, or with dead cells. These mixtures were usually injected immediately, and after incubation at 37° C. for periods of one to four hours.

The rabbits were etherized lightly and at the time of the tracheal injection tilted so that the natural course of the fluid would be into the left lung. Temperatures were taken before tracheal injection and once or twice a day afterward. If not already dead, the ani-

mals, with few exceptions, were killed at the end of forty-eight hours. At autopsy smears were made from the heart's blood and cultures from the pleural cavity, heart, liver, spleen, and trachea. Paraffin sections were made of all lungs, which had been previously distended and preserved in 95 per cent. alcohol or 10 per cent. formalin.

In the first series of experiments preliminary intravenous injection of culture filtrates or dead cells for purposes of sensitization was followed after an interval of two weeks by tracheal injection of living organisms.

Sensitization with Culture Filtrates.

Experiment 1.—A series of 20 rabbits received intravenously 0.1, 0.5, 7.5, or 15 c.c. of culture filtrate of the attenuated culture AA. Two weeks later 1 c.c. of a living broth culture of the same avirulent organisms was injected tracheally. None developed symptoms of anaphylaxis and all were killed after 48 hours.

While there was considerable variation in the lung reaction, in no instance was the lobar type of lesion approached, nor was there any apparent connection between the amount of the preliminary inoculation and the extent and nature of the reaction.

Experiment 2.—Similar preliminary treatment of 8 rabbits with filtrates of the same avirulent organisms, but with the virulent culture substituted in the tracheal injection, resulted in even less lung involvement.

Experiment 3.—In a third series 6 rabbits received both sensitizing and tracheal injections of the moderately virulent, though highly toxic strain B. The febrile reaction was acute. Only one rabbit died within 48 hours, although the lung involvement was much more diffuse and extensive than in the other series. The reaction, however, was apparently independent of the amount of filtrate previously received, and the unsensitized controls developed similar though, on the whole, less marked reactions.

Experiment 4.—In striking contrast was the almost complete absence of diffuse exudative lesions in the series of 16 rabbits receiving filtrates of the virulent strain A followed by tracheal injection of live organisms of the same virulent strain. The animals given the larger sensitizing doses had acquired considerable immunity, as they were still alive after 48 hours, whereas those sensitized with similar doses of the avirulent strain in experiment 2 died.

Sensitization with Dead Pneumococcus Cells.

Experiment 5.—19 rabbits which had received 0.1, 0.5, 7.5, and 15 c.c. of a killed suspension of the avirulent organisms AA were injected tracheally with 1 c.c. of a live broth culture of the same avirulent culture.

While there was more congestion and somewhat more lung involvement than in the corresponding filtrate series, the reactions did not differ materially in the different groups.

Experiment 6.—Eight additional rabbits which had been treated with the same dead cell suspension of the avirulent culture, but injected tracheally with the virulent organisms of the same strain, failed to develop marked exudative lesions. Treatment with large doses of dead cells had apparently a stronger protective action than similar treatment with filtrates of the avirulent culture, as two rabbits in this series were alive after forty-eight hours.

Experiment 7.—The reaction incited by the moderately virulent strain B, when given in both sensitizing and tracheal injections, was studied in 12 rabbits. The majority developed extensive exudative lesions in which areas of hemorrhage and necrosis were quite numerous. In four unsensitized controls, similar, but on the whole less extensive, areas of consolidation were found. The lesions, while more marked, closely resembled those in the corresponding filtrate series.

Experiment 8.—In this experiment in which sensitization with dead cells of the virulent culture was followed by tracheal injection of the same strain in the virulent state, 4 out of 14 rabbits developed extensive exudative lesions, in which fibrin and polymorphonuclear leucocytes were abundant. These lesions, however, were not confined to rabbits which had received the same sensitizing doses. While the two untreated controls died in 24 hours without marked lesions, the protective action of the sensitizing injections was shown by the survival for at least 48 hours of a number of the treated rabbits which had received the larger doses.¹⁰

In the following series concentrated cultures and culture material were used under similar conditions of experiment.

Experiment 9.—Of 16 rabbits treated with the usual concentration of dead cells, 4 received intravenous, and 12 tracheal injections of a concentrated suspension of living virulent organisms grown on agar. None showed anaphylactic symptoms. The lung reaction when present resembled that usually associated with foreign body pneumonias.

Experiment 10.—17 rabbits which had already received sensitizing and toxic injections were reinjected tracheally with a concentrated suspension of pneumococci grown in broth anaerobically under oil. No extensive lesions developed.

Experiment 11.—8 rabbits received single or repeated sensitizing injections of dead cells followed by intravenous injections of a concentrated suspension grown on agar. In one rabbit which had received a single sensitizing injection of 5 c.c., the toxic dose of 2 c.c. was immediately followed by symptoms resembling marked anaphylactic shock followed by complete recovery in about 30 minutes. The lungs at autopsy, 48 hours later, showed practically no involvement. In another series of 7 animals, one rabbit sensitized with 5 c.c. of concentrated dead cells and reinjected with 3 c.c. of a concentrated suspension of live organisms grown on agar died in less than 5 minutes, with symptoms resem-

¹⁰ Additional sensitized rabbits in this or other series died in the latter part of the interval before tracheal injection; a number died of an infection present at the time, but less fatal to normal rabbits, suggesting that the animals, while they had acquired more tolerance to the pneumococcus, were so injured that they had become more susceptible to other infections.

bling anaphylaxis. No unusual reaction was found in the lungs on microscopical examination.

Experiment 12.—For the purpose of comparison 12 rabbits were sensitized with repeated injections of horse serum and reinjected at intervals intravenously or tracheally with the same serum. Anaphylactic shock developed in most of the animals receiving the toxic injection intravenously. Of those injected tracheally one was prostrated immediately after the injection, but recovered in half an hour. Killed 48 hours after the last tracheal injection. The sensitized rabbits' lungs showed possibly more peribronchial reaction than the unsensitized controls which had received tracheal injection, but the difference was not striking and no diffuse lesions of the lobar type were found.

In the preceding experiments active sensitization with filtrates or dead cells of strain A in its highly virulent or avirulent state followed by tracheal injection of live virulent or attenuated cultures of the same race did not result in a definitely increased lung involvement. When a second strain B, of much less virulence, was used, marked exudative lesions, varying to some extent, developed in the rabbits previously sensitized with filtrates or dead cells of the same strain, but the untreated controls also developed similar though possibly less extensive reactions. Since these experiments with active sensitization failed to bring out any very definite relationship between sensitization and the character of the lung lesions, the effect of passive sensitization by means of tracheal injection of different sera and culture mixtures was next studied.

Tracheal Injection of Mixtures of Serum and Culture.

Experiment 13.—Mixtures of 0.5 c.c. of fresh pooled normal rabbit sera and 1 c.c. of living attenuated culture, freshly mixed or incubated, were given tracheally to 8 rabbits. The animals almost at once developed marked signs of discomfort, such as restlessness, dyspnea, and prostration, their temperatures falling at least 3.2° F. The control rabbit, however, receiving 1 c.c. of culture diluted with 0.5 c.c. of sterile broth developed similar, though less marked, symptoms. All recovered in half an hour. The control and the rabbit receiving the mixture incubated for over 3 hours died in about 48 hours. The seven others died within 4 hours of each other in sudden and violent paroxysms, about 24 hours after the tracheal injection. Pneumococci were found in all cultures. A small rabbit injected intravenously with 1 c.c. of a transfer from the culture used in this experiment was unaffected.

The control's lungs showed some peribronchial infiltration. Two rabbits which had received serum mixtures developed extensive, two less marked diffuse fibrinous lesions. Repetition of the experiment failed to incite unusual symptoms, though in the lung of several rabbits small lesions of a fibrinous character were found.

Experiment 14.—Similarly mixtures of normal rabbit sera and virulent living culture were given tracheally to 21 rabbits. No immediate symptoms developed, but a number died in acute paroxysms 24 hours after tracheal injection. In the first series of these rabbits receiving a mixture containing 0.5 serum, which had stood at room temperature for about 2½ hours, one rabbit developed a typical fibrinous lobar pneumonia, the other smaller diffuse lesions. In later series the proportion of rabbits developing diffuse and fairly extensive lung involvement was, however, much smaller. Incubation of the mixtures for 2 to 3 hours appeared to favor a slightly increased lung reaction.

Experiment 15.—Of 12 rabbits receiving mixtures of avirulent living culture and sera from rabbits immunized with culture filtrates of the virulent strain, none showed immediate symptoms, but 7 died within 24 hours of injection—the majority in violent convulsions. The control which had received culture without serum died while not under observation in less than 48 hours. While four serum mixture rabbits developed considerable diffuse fibrinous involvement, the remainder, including the control, showed little or no exudative reaction.

Experiment 16.—The effect of virulent cultures and sera from rabbits immunized with filtrates of the same virulent strain A was tested on 26 rabbits. Of four receiving mixtures which had stood at room temperature 2 to 3 hours and in which the sera was three weeks old, two developed typical fibrinous lobar involvement. In the other series one rabbit showed extensive, and quite a number smaller diffuse fibrinous lesions. While the animals receiving the larger amount of sera possibly showed more lung reaction, the period of incubation apparently exerted little or no effect in this experiment.

Experiment 17.—The experiment was repeated, 11¹ rabbits receiving attenuated cultures and sera from animals immunized to dead pneumococcus cells. As before, the mixtures were incubated for varying periods. All the rabbits were still alive at the end of 48 hours. The lung reaction was distinctly less than in the corresponding normal or filtrate sera and avirulent culture experiments.

Experiment 18.—A series of 22 rabbits was given similar mixtures of virulent culture and sera from rabbits immunized to dead cells. The sera would seem to have exerted a certain amount of protective action, as over half of the rabbits were alive at the end of 48 hours. None developed extensive lesions.

The effect of intravenous inoculation of normal and immune sera immediately before tracheal injection of live culture was tested in the following experiment.

Experiment 19.—11 rabbits were inoculated intravenously with 0.1 or 0.5 c.c. of normal rabbit or horse sera, or with sera from rabbits immunized with filtrates or with dead cells. This was immediately followed by tracheal injection of living virulent organisms. Of the three rabbits, which had received injections of sera from rabbits immunized with filtrates, all died practically 20 hours after injection in acute paroxysms. One of three rabbits, receiving sera from rabbits immunized with dead cells, was alive after 48 hours; the others died while not under observation. None developed diffuse lesions.

The distribution in the lungs of material injected through the trachea, and the resulting injury, must necessarily vary considerably. While these mechanical factors were undoubtedly responsible for certain differences in the bronchopneumonic reaction, they did not obscure the results. Tracheal injections of only 1 or 1.5 cubic centimeters had been given to avoid reactions caused by excessive quantities of concentrated culture such as Winternitz and Hirschfelder had obtained in normal rabbits, and also because it seemed desirable to approach more closely conditions of spontaneous pneumonia in man. Similarly, small sensitizing and non-concentrated toxic doses had been carefully tested, as it was thought a hypersensitive condition favorable to the development of a definite cellular reaction in the lung might be present, although acute or even mild anaphylactic symptoms were entirely lacking.

Active sensitization by previous intravenous injection of pneumococcus filtrates or dead cells failed either to hasten death or to increase markedly the lung reaction, although beginning with as small sensitizing doses as 0.1 of a cubic centimeter the amount injected had been increased until there had been produced an immunity sufficient to protect. In fact, the controls which had not received preliminary treatment were usually the first or among the first to die, indicating that the chief effect of the preliminary treatment was to increase materially the resistance of the animals, especially when large doses were used. While filtrates of the non-virulent culture AA, although originally derived from the extremely virulent strain A, failed, even in large doses, to protect from tracheal injection of virulent organisms of the same strain, and while the protective action of the moderately virulent strain B could not be gauged, as both controls and treated rabbits, with one or two exceptions, live for forty-eight hours, in no instance was death hastened as a result of sensitization.

Comparison of the histological changes in the lungs of treated rabbits and untreated controls also failed to show striking or constant differences, though it appeared that preliminary treatment, especially with dead cells, possibly favored a somewhat more diffuse reaction. In the two experiments in which extensive lesions were found, not only in the treated animals but also to a lesser extent

in the untreated controls, the strain used, though of moderate virulence, was considered especially toxic as compared with the more virulent strains studied. Extremely virulent organisms, on the other hand, apparently encountered little resistance from the lung tissues, but passed immediately into the circulation without inciting exudative reactions. In certain instances where large preliminary doses had been given, the lung involvement was also proportionately greater.

These results would seem to bear out in some measure the view that in pneumonia the lung reaction is partly dependent on a favorable equilibrium obtaining between the natural or acquired resistance of the host and the degree of virulence and toxicity of the organisms rather than on an anaphylactic state.

The reactions, both systemic and local, incited by tracheal injection of serum culture mixtures were somewhat more marked. A supposedly attenuated culture to which normal sera had been added caused immediate but transient symptoms suggesting mild anaphylactic shock. Seven of the eight rabbits, however, died in violent convulsions twenty-four hours later. These acute paroxysms, which closely resembled those of fatal anaphylaxis, also occurred in other series about twenty to twenty-four hours after serum mixtures had been injected.¹¹ The animals suddenly showed extreme restlessness, threw themselves about their cages, making violent running or jumping motions and often crying out. Death occurred within a few minutes of the onset of the symptoms, the heart continuing to beat for some time after respiration had ceased. Examination of the lungs failed, however, to show any definite connection between these paroxysms and the development of exudative reactions.

It is difficult to account for the sudden death of such a large number of the animals at practically the same time, although the interval after inoculation varied in the different experiments, except as the result of some phase of delayed anaphylactic shock. This delayed reaction might be due to several factors, but not to the tracheal method of injection alone, because similar reactions also

¹¹ In other experiments similar results were noted as early as 10 to 12 and as late as 32 to 34 hours after inoculation.

occurred after intravenous inoculation. The capsule of the pneumococcus cell which is protective according to Welch, and the interval required for the production of sufficient toxin, whether from the body fluids or from the pneumococcus cells through growth, might be important factors.

The local tissue reactions incited by the serum culture mixtures, particularly those containing normal serum or serum from rabbits immunized to filtrates, were also somewhat more marked and developed in a larger proportion of animals than in the experiments with active sensitization. Several rabbits developed typical fibrinous lobar pneumonia, and a number of others less extensive but diffuse exudative lesions. These were partly offset, however, by the partial or almost complete absence of exudative reactions in the other rabbits of the experiments, which had received similar treatment.¹²

Throughout the experiments the absence of uniformity in the lung reaction was especially striking. Controls in a few instances developed more involvement than certain of the treated rabbits of the same experiment, while in several series the test animals ran the whole gamut of reaction without apparent relation to the special character of preliminary treatment or tracheal injection.

The extreme sensitiveness of the pneumococcus to any alteration in its environment was repeatedly noticed. While the causes of fluctuation in virulence and toxicity are obscure, they not infrequently seemed closely associated with growth activity. That extremely subtle reactions occur within the body seems probable, and until the laboratory methods more nearly duplicate conditions present in the body, where the cellular elements and body fluids undoubtedly play an important part, similar and constant results can hardly be expected. In the present experiments when body conditions were more nearly approached the lung reaction was generally increased. It is of interest that in Wadsworth's experiment with partial immunization, the pneumococci used in the tracheal injection were grown in equal parts of normal rabbit serum and broth, while Winternitz and Hirschfelder used a 5 per cent. serum medium.

¹² For purposes of comparison tests with heterogeneous sera were made. Three rabbits received tracheally mixtures of culture and sera from a horse immunized to live cultures. Two developed diffuse and fairly extensive exudative lesions, one a very slight reaction.

Although an anaphylactic or hypersensitive condition in the host may possibly enable the invading pneumococcus to gain its first foothold, similar conditions might equally well underlie the development of a streptococcus infection, but would not necessarily determine in either case the type of lesion that later develops. In this study the essentially progressive character of the lung lesion was frequently brought out, all degrees of exudative reaction being found from small peribronchial foci merging into patchy or confluent lesions to the typically lobar involvement.

SUMMARY.

While preliminary treatment with culture filtrates or dead cells of the extremely virulent strain A gave rise to varying degrees of immunity, the exudative lung lesions developing after tracheal injection with live organisms of the same virulent strain were not strikingly increased in any group or series of these rabbits. Despite carefully graduated dosage in the preliminary treatment, none of the animals developed symptoms of a definitely anaphylactic nature.

In similar experiments following sensitization with the attenuated avirulent culture AA, tracheal injection of virulent or avirulent organisms of the same strain failed to incite any definite increase in the exudative lung reaction. In none of the rabbits were symptoms resembling anaphylaxis noted. The immunity which was induced by the larger sensitizing doses of culture filtrates of the strain in the virulent state was lacking when similar doses of the culture filtrates of organisms in the non-virulent state were used.

Extensive lesions developed in both sensitized and unsensitized rabbits when the strain used in the tracheal injection was one apparently combining moderate virulence with exceptional toxicity, indicating that the exudative lung reaction was one of adjustment rather than of acquired hypersusceptibility.

When, in the experiments, small amounts of sera from normal rabbits, or from animals immunized to culture filtrates, were added to the culture before tracheal injection, an increased fibrinous lung reaction was frequently found.

The present study would seem to give some ground for the view that while in pneumonia a hypersensitive condition probably takes

some part in the inception of the infection, the subsequent development of the diffuse exudative reaction in the lung is not directly due to an acquired hypersusceptibility, but to intrinsic qualities possessed by the pneumococcus itself.

This study was carried on in the Department of Bacteriology of the College of Physicians and Surgeons of Columbia University, New York, and later, through the courtesy of Dr. Charles Norris, in the Bellevue Hospital Laboratories. It was completed in the Laboratories of the New York State Department of Health at Albany. I am greatly indebted to Dr. Augustus Wadsworth for his advice and criticism.

THE RATIONAL TREATMENT OF HODGKIN'S DISEASE.*

By J. L. YATES, M.D., AND C. H. BUNTING, M.D.

INTRODUCTORY.

A combined pathologic, experimental and clinical study of Hodgkin's disease extending over a period of six years is the basis of this communication. The most efficacious methods of treatment will be presented with sufficient data to justify the various therapeutic procedures held to be indicated. Fischer¹ was unable in 1901 to find a record of a single case in which cure was *permanent*. None has since then been reported in the literature up to the present year. We have been so fortunate as to have seen two recoveries (that is, five years without evidence of the disease) and at present have a small number of patients under observation who are in a very encouraging condition three to six years after beginning treatment. We believe the disease more common than supposed, more protean in its clinical manifestations than is generally accepted, and not of necessity universally fatal.

This is to be regarded as a statement of observations made and an indication of the results attained by the practical application of the lessons drawn from those observations, laboratory and clinical. Although it is not claimed that a satisfactory routine method of treatment has been established, we feel that a single recovery is indeed encouraging and that prolongation of life, reduction of discomfort and the distinct possibility of 10 per cent. of ultimate cures justifies a conviction that our therapeutic principles are to some extent well founded.

* This work has been aided by a grant from The Rockefeller Institute for Medical Research and by the Trustees of the Columbia Hospital in providing clinical facilities.

1. Fischer: Deutsch. Ztschr. f. Chir., 1901, xxiv, A, 104.

Definition.

Hodgkin's disease may be tentatively defined as a non-contagious, infectious granulomatous process due to the *Bacillus hodgkini*. Primarily localized at or about a portal of entry, it causes a progressive enlargement of a specific nature in adjacent and remote preexisting lymphoid structures, and early provokes characteristic changes in the blood picture. Once well established, the disease manifests little or no tendency to spontaneous recovery.

Etiology.

A pleomorphic diphtheroid organism, gram-positive, antiformin-fast but not acid-fast, has been grown almost constantly,² and usually in pure culture from fresh tissue showing the characteristic pathologic changes in the disease. It has been stained in situ in affected tissue where it may occur in large numbers in an acute process and apparently be the only microorganism present. Subcutaneous inoculation in pure culture into monkeys has provoked a series of changes quite analogous to the disease as manifested in its more acute form in the human. Recovered in pure culture from lesions in a monkey in which the disease had become established, the inoculation of another monkey therewith induced a similar affection as did likewise a subcutaneous transplant of an involved gland from an affected to a normal monkey.³ Moreover, the monkeys soon after inoculation show changes in their blood pictures identical with those observed in spontaneous human cases. Similarly the subcutaneous injection into a quite normal individual of five, ten and twenty million bacilli of a polyvalent stock Hodgkin's vaccine at intervals of five days has produced within four days of the last

2. Negri and Mieremet: *Centralbl. f. Bacteriol.*, 1913, lxvii, 292. Bunting, C. H., and Yates, J. L.: *Cultural Results in Hodgkin's Disease*, *Arch. Int. Med.*, August, 1913, p. 236. Rosenow, E. C., and Billings, Frank: *The Etiology and Treatment of Hodgkin's Disease*, *THE JOURNAL A. M. A.*, Dec. 13, 1913, p. 2122. Steele: *Boston Med. and Surg. Jour.*, 1914, clxx, 123.

3. Bunting, C. H., and Yates, J. L.: *An Etiologic Study of Hodgkin's Disease*, *THE JOURNAL A. M. A.*, Nov. 15, 1913, p. 1803; *ibid.*, Feb. 14, 1914, p. 516.

injection the blood changes which Dr. Bunting⁴ has shown to be an almost constant characteristic of the disease.⁵

Diphtheroid organisms are widely distributed⁶ and are being cultivated from a variety of pathologic tissues. Until the identity of the *B. hodgkini* has been determined accurately it will be impossible to fulfil Koch's dicta to the letter, yet from the facts already established it would seem justifiable to assume that the causal relationship of this organism to the disease has been demonstrated.⁶

Nothing is known of the occurrence of *B. hodgkini* outside the human body. Clinical evidence indicates that it may enter through other portals than those situated in the gastro-intestinal tract. It is at least suggestive in this connection that so-called pseudoleukemia

4. Bunting, C. H.: Bull. Johns Hopkins Hosp., 1911, xxii, 369; *ibid.*, 1914, xxv, 173.

5. Solely for the benefit of the antivivisectionists be it known that the above mentioned individual was a physician who understood the nature of the experiment.

6. It has been so long and so widely held that Hodgkin's disease was of an infectious origin that attempts have been made by many investigators to isolate organisms from tissue obtained at operations or post mortem. Cocci were found first (Verdelli: Centralbl. f. med. Wissensch., 1893, xxxi, 545); later various cocci and bacilli (Brigidi and Peccoli: Ziegler's Beitr., 1894, xvi, 338) leading to a suspicion that a number of bacteria might be causative agents. Delbet isolated a bacillus (Delbet: Compt. rend. Acad. d. sc., 1895, cxx, 1373) which was not described, and was said to have caused characteristic glandular enlargements after intraperitoneal and subcutaneous injections into dogs. Emulsions of glands have been injected into various animals, and if there was not a concomitant tuberculosis the results were quite negative. Thus Longcope produced transient glandular enlargements in monkeys (Longcope: Bull. Univ. of Pennsylvania Med. Dept., 1907-8, xx, 229) which failed to show characteristic pathologic changes. A possible relationship to syphilis has repeatedly been suggested. Spirochetes were said to have been stained in sections of typical glands (White: THE JOURNAL A. M. A., Aug. 31, 1907, p. 774) but this has lacked confirmation though a positive Wassermann reaction (Caan: München. med. Wchnschr., 1910, lvii, 1002) has been obtained in cases giving a negative luetic history. As a heritage of the belief that Hodgkin's disease was an atypical tuberculosis, Fraenkel and Much have held that an organism which they obtained with great regularity from various affected tissues by an antiformin method was a granular, nonacid-fast tubercle bacillus. (Fraenkel and Much: Ztschr. f. Hyg. u. Infektionskrankh., 1910, lxxvii, 159.) They were unable to obtain a culture of this organism or to reproduce the disease by inoculation of animals with the filtrate which contained it. It is probable that they were dealing with *B. hodgkini*.

has been described as having occurred in swine;⁷ in a horse;⁸ in chickens;⁹ in dogs,¹⁰ and in sheep.¹¹ In the last animal the disease is claimed to be infectious and to be transmitted by insects.

General Pathology.

Since the publication of Dorothy Reed's paper¹² twelve years ago, subsequent pathologic descriptions of the more common types of the lesions have been largely repetitions of her observations. With the realization that Hodgkin's disease is probably less limited in its clinical manifestations than was held at that time, and in the light of present conceptions, other less generally accepted lesions should be included in the category. These variations, however, are quantitative rather than qualitative and no radical additions can be made to Dr. Reed's splendid fundamental contribution to this subject.

For example, we have observed two adults showing clinical and pathologic characteristics of a type of lymphosarcoma who had a positive Hodgkin's blood picture, and from whose excised glands an organism identical with or closely related to the *B. hodgekini* was cultivated. In both the disease was first noticed in an enlargement of inguinal glands. Histologically these glands showed an almost complete obliteration of the normal architecture and a widespread proliferation of the "endothelioid" cell particularly marked in numerous foci which on fresh gross section gave an appearance suggesting miliary tubercles.

A man, aged 64, suffering from chloroma in its most typical advanced form, clinical and pathologic, presented a characteristic blood picture, and mediums on which were implanted pieces of excised glands gave pure cultures of what appeared to be the Hodgkin's organism. Macroscopically the greenish tint was evident in a fresh section; microscopically a proliferation of atypical large lymphoid

7. Gunther: Deutsch. tierarztl. Wchnschr., 1906, xiv, 112. Hodgson: Jour. Comp. Path. and Therap., 1903, xvi, 382.

8. Bretegnier: Rec. Med. Vet. Paris, 1909, lxxxvi, 156.

9. Hart: Ztschr. f. Krebsforsch., 1908-9, vii, 485.

10. M'Fayden: Jour. Comp. Path. and Therap., 1903, xvi, 379.

11. Selenew: Folia Haemat., 1911, xii, 99.

12. Reed, Dorothy: Johns Hopkins Hosp. Rep., 1902, x, 133.

cells and the presence of occasional "giant" endothelioid cells and numerous accumulations of blood pigment were particularly noticeable.

Still more surprising were the results of observations on a young physician who had for seven years been suffering from periodic attacks of a peculiar type of subcutaneous inflammation associated with pain, fever, delirium, leukocytosis, lymphangitis, and lymphadenitis affecting one leg and causing a very gradual enlargement suggesting elephantiasis. His blood picture was that of Hodgkin's disease both between and during attacks. Cultures taken from obviously involved subcutaneous fat and from enlarged popliteal glands developed an apparently characteristic diphtheroid organism. Sections from the fat showed a definite perivascular inflammation, a mononuclear cellular infiltration and fixed tissue proliferation. Those from the glands showed a distortion of architecture, proliferation of endothelioid and lymphoid cells with scattered endothelioid giant cells and with a fine, diffuse, fibroblastic proliferation. A periadenitis of character similar to the perivascular inflammation in the fat was also present. Eosinophilic infiltration was lacking.

The successful cultivations of a similar organism from the spleen pulp after splenectomy in two cases in which a clinical diagnosis of early Banti's disease had been made are very suggestive, particularly since it has been held (Westphal) that there is a purely splenic type of the disease. In these instances the blood picture was not quite positive because of the absence of an increase in the size and number of the platelets. Microscopically there were the usual increase in stroma, destruction of the malpighian corpuscles and excess of the endothelioid cells. The cultivation of a characteristic organism from the glands in a case of lymphatic leukemia by Steele and the observations of the Fraenkel-Much granular bacillus in this disease are suggestive, but only suggestive, of a possible relationship of these diseases and not proof of their identity.

A middle-aged woman with moderately advanced arthritis deformans had, in addition to carious teeth wonderfully masked by most artistic crown work, obviously affected tonsils and enlarged inguinal glands. Cultures made from these glands developed a diphtheroid bacterium in pure culture, a section therefrom showed

histologic changes indicative of Hodgkin's disease and virtually conclusive additional evidence was in the changes in her blood picture. As a corroboration of these findings may be cited the observation of another patient suffering from Hodgkin's disease in a subacute form who has had, but only since the affection became well established, recurrent attacks of multiple arthritis apparently attributable to no other infection.

Certain erroneous statements are frequently encountered, quotations from observations made when pathologic and clinical diagnoses differed from present standards. Some have a definite therapeutic significance and should be refuted.

It is incorrectly maintained that the capsules of lymph glands are never penetrated and that the periglandular tissues are therefore not directly invaded by the intraglandular granulomatous process. This capsular penetration is unusual and is only marked when the disease is progressing acutely. It has also been held that there is no periadenitis, hence no matting together of the glands or formation of adhesions to the skin or deeper structures. This too is incorrect. A periadenitis is virtually constant, probably developing from a lymphangitis as dissemination occurs, indeed virulent specific organisms are to be found in the periglandular tissues. These periglandular tissues, as pointed out by Fischer, are moist, vascular and friable in acute or recent involvements and are more apt to be dry, avascular and firm in the older and more chronic varieties. Thus, without evidence of secondary infection, intimate and dense adhesions to the skin occur and rarely may even lead to ulceration, according to Delbet.¹³ The brawny induration of the skin present in several of our cases indicated that certain of the board-like subcutaneous inflammations might be due to the *B. hodgkini*, and this has been already confirmed in a case cited above.

Likewise more or less conspicuous and dense adhesions are found between the glands themselves and surrounding deep structures, often leading to an erroneous conclusion that a malignant type of infiltration has been encountered. The fluctuations in the size, consistency and degree of mobility so often noticed in the course of the

13. Delbet: *Semaine méd.*, 1893, xiii, 430.

disease are probably attributable to a large extent to waves of intensity in this periadenitis.

Contrary to a frequently repeated statement, foci of macroscopic softening do occur, without any secondary infection, though but rarely, and then particularly in the more acutely affected glands. When the process is older or more chronic there is a correspondingly increasing sclerosis which virtually becomes complete if life is sufficiently prolonged. Even when there is little to be found microscopically except fibrous tissue the organism can be cultivated with proper technic.

Living Pathology.

Since the living pathology of any disease is a foundation for its therapy an attempt will be made to portray that of Hodgkin's disease as we have come to see it through superimposed pathologic and clinical spectacles.

When Wilks gave to Hodgkin's disease an intermediate position between cancer and tuberculosis he did all three an injustice. Hodgkin's disease may be said to possess all of the evil clinical characteristics and none of the virtues of both a malignant neoplasm and a malign infection. By the latter is meant an infectious process in which the balance of power between virulence and resistance is so delicately adjusted that it may be disturbed by factors frequently unrecognizable and usually negligible under other conditions. In the constant struggle for supremacy the powers of resistance ultimately succumb, but only after repeatedly having overcome the temporary advantages gained by relatively or actually increased virulence.

The offensive mechanism may be regarded as both neoplastic and inflammatory *in its effects*, attributing to the former, more particularly, the mechanical or physical injuries and to the latter more especially the toxic or physiologic.

With the establishment of a portal of entry, a lymphangitis partly toxic and in part due to the presence of bacteria marks the first extension of the disease toward the next lymphadenoid station, there to provoke a specific lymphadenitis. Perilymphangitis and perilymphadenitis are significant and constant features in the proc-

ess. The entire progress of the disease is a repetition of similar steps, now more acute, now less, increasing numerically here by arithmetical there by geometrical progression, almost never constantly but in alternating waves of progression and regression due probably to periods of disproportion between the powers of virulence and resistance, and varying directly with the degree and duration of this inequality, being at times well localized, at others quite general.

Thus may be explained at the one extreme the extension between glands or groups of glands without any notable concomitant reaction, and at the other extreme those characteristic recurrent febrile attacks.

Three stages of the disease may thus roughly be outlined :

Stage 1. Process localized, physical effects slight, physiologic effects usually unnoticed, though a positive blood picture, an expression of toxemia, is already developed.

Stage 2. Considerable dissemination, physical effects more marked but still relatively insignificant, physiologic effects now definite, toxemia and anemia.

Stage 3. Wide dissemination, physical effects pronounced, edema, dysphagia, dyspnea, physiologic effects advanced, toxemia, anemia and cachexia.

Manifestly, in any given case, the variations in extent and intensity of the physical and physiologic effects present diverse problems in diagnosis, therapeutics and prognosis.

Apparently local and general resistance may be estimated to some extent histologically. The end-result so far as local recovery is concerned is fibrous tissue. An index of the general resistance can be found in the blood picture where there seems to be some direct significant relationship between a normal proportion of small lymphocytes and a favorable prognosis.

Sarcomatous metamorphosis in Hodgkin's disease has been described by Yamasaki,¹⁴ Karsner¹⁵ and Symmers.¹⁶ A necropsy in

14. Yamasaki: *Ztschr. f. Heilk.*, 1904, xxy, 269.

15. Karsner, H. T.: *A Study of Cases of Hodgkin's Disease and Certain Allied Conditions*, *Arch. Int. Med.*, August, 1910, p. 175.

16. Symmers: *New York Med. Jour.*, 1911, xcii, 971.

the most acute and extensive case we have seen showed the parietal pleura, visceral pericardium, intercostal and pectoral muscles to be riddled with nodules. Macroscopically the appearance of malignancy was as distinct as was the picture of typical Hodgkin's granulomatous infiltration, microscopically. The possibility of malignant metamorphosis must be admitted, also its improbability, inasmuch as the causative agent is to be found in and to be cultivated from these so-called malignant metastatic lesions.

Confusing complications of secondary infections, other than agonal, occur and may make a certain diagnosis extremely difficult. When tuberculosis is engrafted on an established Hodgkin's process, as not infrequently occurs late in the disease, the histologic diagnosis is simple. Exceptional cases are reported, as cited by Fischer and Dietrich,¹⁷ in which the two diseases were thought to have developed simultaneously. These should present little difficulty of recognition from a pathologic point of view at least. If any such cases have come under our observation they have been unrecognized. The greatest difficulty has been encountered when, apparently, Hodgkin's disease may have been engrafted on an old tuberculous adenitis in which there exists both caseation and secondary acute infection. We have two such cases under treatment. Glands obtained at two operations on bilateral cervical enlargements of one individual showed advanced tuberculosis with much caseation and some less chronic changes that could not with certainty be identified. Cultures give cocci, *B. subtilis* and in one tube a diphtheroid bacillus which could not be isolated. Definite evidence of an acute infection grafted on a Hodgkin's disease has not yet been established in our work.

Clinical History.

Heredity and contagion are without apparent significance, though a case cited by Albutt is still quoted as indicative of the latter being a possibility. Unhygienic surroundings may have some influence, particularly deficient personal cleanliness. No age is exempt, and contrary to the usual statement the sexes are about equally affected, though in males the disease is apt to develop in younger individuals.

17. Dietrich: Beitr. z. klin. Chir., 1896, xvi, 377.

Almost every conceivable condition supposed to reduce resistance has been suggested repeatedly as a predisposing factor. It is an interesting coincidence that diphtheria has been mentioned but once in the literature to which we have access.¹⁸ Robust young persons are attacked while in apparently excellent health; more often perhaps, careful inquiry will disclose symptoms of some minor affection that will offer a satisfactory explanation for the onset or at least for the entrance of the infection.

The predilection of Hodgkin's disease to manifest itself primarily in an enlargement of the cervical glands led Trousseau to investigate all structures drained by these glands as possible portals of entry for infection. Murchison first directed attention to the tonsils which appear now to be the worst and most constant offenders. Not only may the disease be limited for a time to enlarged tonsils, as in a case reported by Delie,¹⁹ and have a possible origin from a frank tonsillitis (Westphal), but it can affect these structures so slightly as to give no external evidence of abnormality. We have seen typical histologic Hodgkin's changes in tonsils which appeared normal clinically. Locating the disease in the tonsil is not sufficient, as this may be a secondary infection from other sources in the mouth and nasal passages. The possibility of carious teeth being portals of entry was recognized by Gowers. Rosenow has cultivated the *B. hodgkini* from peridental abscesses and sinuses.

In a fatal case with acute ethmoidal sinusitis, Bunting obtained this organism in pure culture from the affected cells. Organisms morphologically identical with those obtained in gland culture were found in smears made from secretions obtained from the upper nasal passages in a case in which the disease was present in the cervical glands. Chronic dacryocystitis, rhinitis, pharyngitis and otitis media are other lesions which have apparently been the starting point of the disease. We have seen two cases in which the portal of entry was certainly intestinal and others in which this was a possibility. In another the disease may have begun in the bronchial glands. Fraenkel and Much believe the infection may take place through the respiratory tract.

18. Westphal: Arch. f. klin. Med., 1893, li, 83.

19. Delie: Rev. hebd. de laryngol., 1908, ii, 499.

Preexisting adenitis has been noted frequently. Peacocke²⁰ observed the development of Hodgkin's disease in twin boys aged 4, both of whom had had measles complicated by adenitis. Other children in the same family had had measles but without adenitis. Delbet cites a case attributed to Billroth, in which the disease supervened on a lymphangitis and adenitis due to a bee sting. The possibility of this infection being added to a tuberculous adenitis has been mentioned above.

One patient with a primary enlargement of her inguinal glands had a profuse leukorrhea. Smears from the mucus from her cervical canal showed in considerable numbers an organism similar to that obtained in pure culture from an excised gland. Another had a sharp attack of cystitis preceding the onset, which is of greater significance since Fraenkel and Much observed an infiltration of ureter and bladder with Hodgkin's disease that they attributed to renal infection.

Skin lesions are fairly common. Fifteen per cent. of the cases studied by Westphal showed some skin affection and in 10 per cent. a lichen-like eruption preceded the onset. Trousseau's statement, so often repeated, that chronic irritations of the skin and mucous membrane may give access to a Hodgkin's infection, is evidently upheld. The site of the primary glandular enlargement would give a fairly definite regional location of the actual portal of entry.

The course of the disease depends on the relative virulence of the infection. In the acute forms death may result in a few (two to four) months; in the most chronic, life may be prolonged up to five years. A feeling of lassitude may precede recognition of the disease but more often the glandular enlargement is a fortuitous observation when the general health is good, notwithstanding there has been an absorption of sufficient toxin to alter the blood picture. During the early stage while the disease is localized in the primarily affected group of glands there is so little effect on the general health that proper attention is often neither sought nor given when it is sought. In acute cases there is a rapid transition into an intermediate stage where dissemination is manifest, physical and mental depression more constant and anemia, due to a toxic

20. Peacocke: *Tr. Roy. Acad. Med., Ireland*, 1905, xiii, 1.

hemolysis, becomes evident. This transition into an intermediate stage in chronic cases is usually gradual and marked rather by the accidental discovery of additional glandular swellings more and more remote from those first affected. Exceptionally, following a period of chronicity there is a sudden explosive dissemination, after which a more acute course is run, suggesting a very rapid diminution in the powers of resistance of the individual. One of the most strikingly characteristic features of the disease, to be seen in the intermediate stage particularly but occurring if less obviously in the others, is the alternating periods of exacerbation and remission in the intensity of the process. During the periods of progression, the conditions manifested by the acute forms are simulated somewhat, there is more effect on the general health, the groups of glands become larger and the individual glands therein, less differentiable and movable and a transient sensitiveness may develop. The overlying skin is less movable, may be boggy and even become reddened. These periods are relatively short and escape the notice of unobserving patients, though most all can recall the periods of remission when there is generally a marked diminution in size of the glandular tumors. This decrease in size may be so great and so persistent and so often accompanied by a sense of well-being that any treatment then in vogue, though the same improvement may be entirely spontaneous or follow an intercurrent acute infection, is apt to be considered causative, and another cure for Hodgkin's disease thereby discovered and unfortunately is usually promptly reported. Sooner or later another recrudescence appears, usually more vicious than those preceding, but now uncontrollable by the measures formerly so efficacious, and not infrequently introducing a rapid, final decline. Those later details, however, quite constantly escape publication.

During the intermediate stage extension takes place from one cervical triangle to the other, then to the neighboring axilla, the opposite side of the neck, the mediastinum, the opposite axilla, the spleen, the groins, the liver and retroperitoneal glands, in about that order. When the involvement has become widespread, the effects of increased intoxication and of pressure make themselves evident in the terminal stage. Here is noted the grave anemia that may

simulate the pernicious type, and the marked cachexia and emaciation, the tendency to purpura and to repeated hemorrhages from the mucous membranes due to a deficiency of blood platelets resulting from exhaustion of the bone marrow, febrile attacks, occasionally of the relapsing (Murchison) type, pleural and pericardial effusions, ascites, obstruction to blood and lymph circulation and to respiration. It is during this late stage that miliary tuberculosis so often develops and is unrecognized until revealed at necropsy.

No description can be given of the nature and progress of the less common types of Hodgkin's disease that are not associated with glandular enlargements nor those varieties that are just beginning to be recognized as such through bacteriologic and hematologic methods.

Diagnosis.

It is impossible to escape a recognition of the very pronounced similarity between the clinical aspects of Hodgkin's disease and those of many malign neoplasms especially in the tendency to dissemination and to recurrences, and the ultimate general effects on the individual. In no disease is a prompt and particularly an early positive diagnosis of more consequence in successful treatment. This most important part of the therapeutic problem has, we believe, become relatively simple.

Without detracting in the least from the great importance of accurate history taking with its proper interpretation and a most thorough and complete examination,²¹ these observations alone will not lead to an indisputably positive recognition of the disease, at

21. A complete examination will determine the details of treatment and should include careful investigations by trustworthy specialists of the mouth, throat and nose, including the accessory sinuses, and may be satisfactorily conclusive only after skiagraphs have been made. No thoracic examination, however carefully done, is adequate until suitable roentgenograms have determined the absence of involved mediastinal glands or their size and location if present. When there is slight involvement in the anterior mediastinum, it is often unrecognizable by other methods, and this is especially true of the posterior which is invaded more frequently than is supposed. Moreover, this affords the only accurate method of gaging the efficacy of treatment even when the intrathoracic involvement is sufficient to be recognized by ordinary methods of examination. No great concern need be given to actual lung involvement as this is a manifestation of a late and probably a hopeless stage.

least while a cure is still possible or probable. Final proof comes from the laboratory and there must be a competent objective observer who can recognize and construe slight variations, hematologic, bacteriologic and histologic. At the present time a satisfactory blood examination gives the most reliable immediate diagnostic evidence. Histologic studies cannot be equally positive until the early minute pathologic appearances of borderline affections are definitely established, granting that this may eventually be accomplished, and can never be possible in those less common types which do not manifest glandular swellings or easily accessible involvement in unimportant tissues. Nor can bacteriologic methods be uniformly satisfactory. Diphtheroid organisms are ubiquitous. Contaminations may occur under the most rigid technic, and more important still, the organism may take ten days to grow satisfactorily.

Material for histologic study can never, and for bacteriologic study, seldom, be obtained without the excision of a test gland or some affected tissue. Personal observations would have convinced us, even were the proof not clearly written in the reported experiences of all who have treated this disease, that partial excisions were accompanied by such actual and not hypothetical dangers of dissemination that "test excisions" are absolutely contraindicated unless the stage or the character of the disease is such that the only hope lies in vaccine therapy, should its value ever be undisputably established. Even under these conditions, using a polyvalent stock vaccine may be a safer method than taking some undue chances to obtain an autogenous culture.

Since Bonfils (1856) first pointed out that there was no increase in the white blood corpuscles in Hodgkin's disease and this fundamental observation was incorrect, it has been held that there are no characteristic blood changes in this affection, and that such as were observed marked either a secondary infection or a transition to lymphatic leukemia.

The essential features in the blood picture that have proved to be almost pathognomonic with but few conditions to be excepted, can be summarized briefly.²²

22. It is quite probable that the reasons why these characteristic variations in the blood picture have so long escaped detection, depend on the technical methods

There are two distinct types, an early and a late, showing a constant characteristic increase in the number of platelets (unless exhaustion of the bone marrow occurs) with abnormally large forms and either a relative or absolute increase in the so-called transitional cells. In the early type the leukocytes are usually less than ten thousand. Very early there is a moderate increase in the basophils, and when the disease is well established the eosinophils are slightly increased. The polymorphonuclear neutrophils remain within the usual limits, the lymphocytes at or slightly above the normal. The late type shows a leukocytosis which may reach one hundred thousand, the neutrophils are relatively increased to a percentage of from 75 to 92 and the lymphocytes are reduced to 5 per cent., frequently even less. The transitionals, the only other cells found in any numbers, are usually above 8 per cent. unless the leukocytosis is very high, when they may be relatively fewer, but still exceed the lymphocytes in number. The late picture is a result of the intensity of the disease rather than the extent of involvement and is usually largely dependent on duration for its development. Not only has this blood picture held clinically, having been confirmed in every instance either histologically or bacteriologically or both, but has appeared in monkeys inoculated with the living Hodgkin's bacillus and in one normal individual injected with the same, but killed, bacteria. Notably in one case of chloroma and two of so-called lymphosarcoma, the blood examination was positive and was con-

followed in making and staining the blood smears and on the varying conceptions of the proper differentiation of the groups of leukocytes. Thus Litten is quoted by Fischer as having noted the platelet increase in 1895, an observation which Fischer attempted unsuccessfully to confirm, but which was upheld by Selenew who also remarked the very large forms. It remained for Bunting (*Bull. Johns Hopkins Hosp.*, 1911, xxii, 114) to show the constancy and evident source of these structures in Hodgkin's disease. Lymphocytosis occurs and has been recognized by many observers, but is of little diagnostic importance since there is a constant and marked lymphopenia after the early stage. This was commented on by Steiger (*Berl. klin. Wchnschr.*, 1913, i, 2129) who also repeated the old observation of a tendency to eosinophilia, which may be so high (over 20 per cent.) as to suggest the presence of some animal parasite and yet be due, as Lincoln's case has shown (*Lincoln: Boston Med. and Surg. Jour.*, 1908, clvii, 677) entirely to an atypical manifestation of the disease. The leukocytosis which occurs in the late stages has been noted repeatedly.

firmed by the cultivation of typical organisms from tissues that histologically alone might not have been properly diagnosed.

Confusion has arisen so far in but one class of cases, those of tuberculous adenitis with an acute (coccal) secondary infection. Here, too, histologic and bacteriologic controls can fail to be conclusive. Differentiation from uncomplicated tuberculous adenitis is seldom difficult, the blood pictures are distinct and the reaction to tuberculin usually confirmatory. We have seen a positive von Pirquet reaction but four times. One patient had a clearly definite apical lesion, one, an adult with so-called lymphosarcoma, was without a demonstrable tuberculous focus, and the other had preexisting tuberculous adenitis. In an acute case miliary tuberculosis was discovered post mortem and there had been a negative tuberculin test two months before. This man showed shortly before death a temporary transition to what is commonly accepted as a lymphatic leukemia blood count.

Treatment.

However constant has been dispute as to the name or nature of the disease, there has ever been unanimous agreement that it was inevitably fatal in less than five years. Evidently, therefore, successful therapeutic measures would have to differ from any hitherto employed and must find their application in combating the various pathologic processes manifested as the disease progresses, while attempting at the same time to protect and enhance such curative physiologic reaction as could be recognized clinically and pathologically. Five years elapsed after our investigations began before the *B. hodgkini* was first isolated and its probable etiologic relationship established, so that we had opportunity to try other therapeutic methods than those dependent on a specific bacteriology. Our working hypothesis has been to accomplish (1) an exclusion of all possible liability to reinfection, hence a removal of the primary source of infection and its portal of entry; (2) the most rapid elimination of at least the major portion of the diseased tissue with the bacteria and toxins contained therein, in order to prevent further dissemination and to place the balance of power in favor of individual resistance, wherefore its surgical extirpation when con-

ditions permitted; (3) a destruction of the remaining bacteria by any and every means, especially the Roentgen ray and hygiene, and more recently vaccine therapy has been employed; (4) the conversion into fibrous tissue by hygiene and the Roentgen ray of such irremovable abnormal tissue as cannot undergo resolution; (5) and finally, to continue treatment as circumstances seem to indicate until a clinical and roentgenologic examination and a normal blood picture indicate a cure has been of at least a year's duration.

The measures to be described hereafter have been found to give the best results, results so encouraging at present that their presentation seems to be justified. Every detail has been found to be of such importance that the reasons for the application of each will be given lest any be considered unessential and be slighted or discarded at the cost of the utmost possible prolongation of life and comfort of some individual. It should not be supposed that the procedures are equally applicable to all cases whatever be the stage of the disease or evident virulence of infection. Here again be it known that the extent of involvement is less significant than its location and nature, which can only be revealed by the detailed examination already outlined.

1. *Removal of the Source of Infection.*—A. Where involvement is primarily cervical a complete pericapsular tonsillectomy is definitely and invariably indicated even if the tonsils appear perfectly normal. In addition the teeth, nose, ears, eyes and accessory structures must be excluded as the site of primary infection, or properly treated. B. If the primary glandular involvement is extracervical, a search must be made for a source of infection in the area thereby indicated and suspicious lesions treated appropriately. These measures should be instituted immediately and when possible carried out under local anesthesia, observing, so far as possible, the principles underlying the treatment of malignant tumors.

2. *Extirpation of the Major Portion of the Disease.*—Excision of the cervical glands can be done as thoroughly as in the block dissection advocated by Crile, with the possible exception of preserving at least a part of the eleventh nerve and observing otherwise all of the precautions advocated by Halsted to safeguard dissemination. This can be accomplished without unduly reducing resistance.

and we believe with less risk to the individual, than attempting to treat them otherwise. Any dissection less complete and careful is positively contraindicated.

The glands will be found to be more or less adherent, in some instances so adherent that their separation from the subclavian vein will be difficult, from the internal jugular so formidable, that this vein should be excised unless there be any question of the patency of its fellow. Glands adherent at the subclavian-jugular angle may be extremely trying to extirpate, but their removal is imperative in that this last source of mediastinal infection must be excluded. In spite of every effort we have cut the thoracic duct in two individuals. In one it was ligated, in the other the discharge of chyle was finally controlled by pressure. Neither manifested any ill effect.

The entire area exposed should be treated thoroughly and actively with full strength tincture of iodine and drained, as it is inconceivable that all contamination can be avoided. This additional irritant also increases the immediate inflammatory reaction, a very desirable feature, particularly as it has no effect on the ultimate functional or cosmetic result. Exposures of the entire wound area to the Roentgen ray should be started not later than the second day, preferably within a few hours of operation and continued at intervals of a few days to the limit of skin tolerance, for reasons that will be given later.

Such radical treatment will be criticized particularly by those wanting in surgical sympathy. In explanation be it known that any less thorough or even as thorough excision, without disinfection and subsequent radiation, may be followed by a recurrence, which, as Reed has reemphasized, can be more extensive than the original involvement. Recurrences in Hodgkin's disease are as little to be desired as in carcinoma. We have seen other glandular enlargements (for example, axillary, opposite cervical) begin their final decline *only after the removal* of this source of infection or drain on the powers of general resistance when the treatment was otherwise unchanged, just as we have seen some cervical enlargements permanently disappear under the Roentgen ray *after* tonsillectomy. These observations have been assumed to indicate that the balance of power has been changed and have been sufficiently numerous and

well controlled to convince us that such treatment may determine the ultimate result no matter what other therapy is employed.

As yet we have not seen the same beneficent effect on mediastinal involvement, though apparently complete and final resolution has followed in axillary glands that have been probably, in size, at least, as extensively involved. The explanation seems to lie in the comparatively slight effect of the rays on intrathoracic tissue. Progress in intrathoracic involvement has been checked in two individuals if the radiographic evidence is conclusive. The general health has remained unimpaired in one boy for over six months and one girl appears radiantly healthy after four months, but in both the blood findings are ominous. Mediastinal infection may occur early or be absent even when both sides of the neck and both axillae have shown extensive enlargements for months. The appearance of intrathoracic involvement, in our experience, has been of more sinister significance than a moderate splenic or hepatic enlargement. Indeed, these may indicate, when other conditions are favorable, but an extensive reaction on the part of the defensive mechanism. Therefore we emphasize the extreme importance of protecting the mediastinum from any infection by extension from the neck or axilla, particularly before, but also even after intrathoracic involvement has occurred. We take this to mean that when the excision of the primarily affected cervical glands is not followed promptly by the progressive resolution of secondarily enlarged glands which may infect those in the mediastinum, that these glands also should be excised.

Axillary dissections should be as extensive and as thorough as the cervical. This requires division but not excision of the pectoralis major and resection of the minor. The nerves supplying the pectoralis major and the long thoracic nerve should be preserved. Involved glands have been found anterior to the major pectoral, between it and the minor and extending upward along the vein to the falciform ligament. An incision can be made along the anterior axillary line, extending thence if necessary a short distance along the lower margin of the clavicle that will give adequate exposure and not injure the breast tissue in women nor be cosmetically ob-

jectionable. Subsequent wound treatment should be the same as after cervical excisions.

No case in which the inguinal glands were affected primarily has come at a stage when radical extirpation was thought to have been indicated. The same general plan should be followed to protect the retroperitoneal as the intrathoracic lymphatics.

Patients presenting themselves in the later stages of the disease, with advanced involvement, intrathoracic or intraperitoneal or both, have been subjected to no radical treatment and this has probably been a mistake, at least in exceptional cases. Those in fairly good general condition and in whom the blood changes do not indicate the latest stage may be benefited materially by thus starting a regressive phase when other methods may help greatly. Without the initiative of this wave of regression those procedures have been futile. In one apparently hopeless case the patient has shown remarkable progress during the period between the excision of glands and the final preparation of her vaccine. This particular organism grew very reluctantly, and the excision removed considerable tissue from neck and groin because the periglandular adhesions made safer and easier the extirpation of groups of glands. Had she received any treatment in this interval there might be less justification in attributing some causal relationship to this excision.

3. *Destruction of Bacteria by the Roentgen Ray.*—No permanent cure has ever been obtained solely by the use of these rays. Numerous instances of marked improvement have been noted since the observations published by Senn.²³ Pfeiffer²⁴ made a very careful and extensive study of the value of Roentgen therapy in Hodgkin's disease and found the usual discouraging facts; after varying intervals of great improvement recurrences were inevitable and finally became uncontrollable. The reasons for its marked temporary efficacy have, to some extent, been discovered. A very resistant culture of *B. hodgkini* implanted on the most favorable mediums was destroyed by one exposure under conditions identical with the therapeutic dose, so long as these cultures were not much over 5 days old. Later than this the involution forms that develop so

23. Senn : New York Med. Jour., 1903, lxxvii, 655.

24. Pfeiffer : Beitr. z. klin. Chir., 1906, 1, 277.

characteristically are more resistant, and after exposure to the ray the control subcultures were positive. This effect is not due to changes in the mediums, which after the same exposure supported subsequent cultures apparently equally well.

The patient from whose tissues the *B. hodgkini* was first grown in 1912, had, low in her right axilla, two glands a few inches apart. One was excised as a control. Cultures were positive, sections showed the usual moderately chronic process. The other gland was subjected to the usual radiotherapy and later excised.²⁵ Cultures this time were negative, when the technic had been developed so that positive cultures were being obtained constantly from tissue that was found histologically to show the disease. Comparison of the sections, which unfortunately were not stained and studied for the presence of bacteria, showed increased sclerosis, hyaline degeneration and an abundance of the "endothelioid" cells in excess of what could have been expected by natural reaction in the elapsed time. Moreover, there was present in the periglandular tissue, increase in fibrous elements as well as infiltration with round cells and neutrophils.

The effects of the ray immediately after operation are of the same character, only possibly less intense, than when given after excisions of malignant neoplasms with reflected skin flaps as advocated by Crile. There is a remarkable increase in the inflammatory reaction shown by the excessive serous exudate and superficial deposition of fibrin, the overlying skin is edematous and healing correspondingly delayed. After several months the evidences of the excess irritation disappear completely, the skin becomes soft and movable, pigmentation disappears, the scars of incisions are narrow and pale and there is a much reduced liability to keloidal changes.

Burns have resulted exceptionally and are probably not always to be avoided even under the most skilful administration and accurate dosage of the rays. We have been singularly fortunate in having the cooperation of exceptionally competent therapeutic roentgen-

25. Invaluable lessons were learned from this patient who, with the one exception noted below, is the last who has been subjected to meddlesome surgery. As a result of partial excisions the disease was so spread in the axilla as to demand a complete axillary dissection to protect the mediastinum, which has fortunately remained free.

ologists who will subsequently make a formal report of their work and observations. It is a pleasant duty to acknowledge an indebtedness to Dr. Foerster and Dr. Baer for their unflagging interest in our work and generous care of these patients, but particularly for having proved the value of this phase of treatment, possibly as important as any other detail, when given rationally as to time, place and method.

4. *Hygiene*.—Whatever treatment may be selected, evidently the native individual resistance will be the fundamental factor in determining the outcome. Nothing that will improve general physiologic efficiency should be omitted as everything should be that might provoke physiologic deficiency. In this connection may be considered the question of drugs. Tonics are often indicated. Arsenic is no more specific than iron. The uses of maximum doses of Fowler's solution internally, subcutaneously or injected directly into the affected glands has been wondrously exploited. Its value is not more than transient. Marked and prompt improvement has followed the administration of salvarsan intramuscularly and intravenously, as in a case treated by Galland,²⁶ another treated by Dr. L. F. Barker (personal communication) and in one of our series, but these effects are only fleeting and when recrudescence appears it is unaffected by further administration of this remedy. No indication has been found for the use of benzol (benzene C_6H_6). The treatment of one case by this means has been recorded,²⁷ but the diagnosis was not established and only the early results were reported.

The mixed toxins of erysipelas and *Bacillus prodigiosus* so warmly and authoritatively advocated by Coyle have not been tried, in spite of the knowledge that almost complete if temporary disappearance of glandular swellings has followed acute intercurrent infections, notably in one rather advanced case after a severe scarlatina. The belief that Hodgkin's disease was an inflammation, and a disinclination to add to the intoxication in view of the lack of evidence that any permanent benefit has thus been obtained, even

26. Galland: Med. Press and Circular, 1911; New Series, xcii, 43.

27. Lawson, G. B., and Thomas, E. A.: A Case of Hodgkin's Disease Treated with Benzene, THE JOURNAL A. M. A., Dec. 13, 1913, p. 2157.

in Coley's cases,²⁸ were considered sufficiently contraindicated. In a personal communication Dr. Coley has stated that a patient under the care of a former assistant was treated in the Ottawa hospital and has remained well for six years.²⁹ In this instance, while the clinical diagnosis appeared probable it was not confirmed pathologically and must therefore be, to some extent, questionable.

5. *Vaccines.*—Successful active or passive immunization in the light of the constant failures that have followed all other therapeutic methods, has been a stimulating hypothetical possibility. Encouraging results are not wanting, neither are the discouraging, at least so far as vaccination is concerned. Improvement has followed subcutaneous injections of both polyvalent stock vaccines and the autogenous, but not in every case, and in some at least, the same old discouraging sequence of events ensues and subsequently an uncontrollable recrudescence leading rapidly to death gives the impression that history is repeating itself and that another "cure" may have to be admitted to the list of failures.

In no case that we have seen treated by vaccination has there been a favorable result in the rapidity, extent or permanence of improvement that has not been equalled at least by observations on similar cases treated essentially similarly except for the vaccine administration.

One boy whose history will be given has had vaccine treatment over a period of two months for a slight mediastinal involvement, the only discoverable manifestation of a recurrence. Stock vaccines prepared by Dr. Rosenow, who generously forwarded an adequate supply, and by Dr. Clara Moore at the University of Wisconsin according to Dr. Rosenow's directions, have been given at five-day intervals, to the total number of 62,000 million bacilli. There has never been a local or general reaction and his health has remained good. The involvement has remained stationary but in addition he has been receiving the Roentgen ray in maximum doses. His blood is beginning to show features of the late stage.

Another patient, whose history also will be given, had, in addi-

28. Coley: *Am. Jour. Surg.*, 1908, lxxvii, 127; *Surg., Gynec. and Obst.*, 1908, vi, 649.

29. Coley: *Surg., Gynec. and Obst.*, 1911, ix, 174.

tion to extensive mediastinal involvement, a small group of involved glands in her right axilla. She received subcutaneously over a period of four months 18,000 million bacilli of an autogenous vaccine. There was improvement in her general health, increase in weight, but not more than could have resulted without the use of vaccines. No marked general reactions resulted at any time, though subcutaneous nodular induration appeared at the sites of the injections. Roentgenographic and physical examinations showed no diminution in the mediastinal disease and her blood picture remained unchanged. It became essential to know what had occurred in the axillary glands as they had not changed noticeably in size or consistency. A partial excision was done and the glands were removed, followed by the maximum doses of the Roentgen ray which had not hitherto been given at this area. These glands were not manifestly different microscopically or macroscopically from those obtained from her opposite cervical region, three months before; in fact, the microscopic pictures were virtually identical.

There is no desire to detract from the possible value of vaccine therapy in Hodgkin's disease, but such observations as we have been able to make, and every effort to obtain proper controls and to maintain an objective point of view has been exerted, will permit of not more than a Scotch verdict. Vaccination in our hands cannot be shown as yet to have exerted marked if any influence on the ultimate course of the disease, but admittedly it is too soon to pass final judgment. If there is an ideal method of vaccination for Hodgkin's disease we believe it is yet to be established.

6. *Conversion of Abnormal Tissue into Fibrous Tissue.*—This is desirable when resolution is impossible, in order to remove any tendency to pressure effects and the liability to subsequent infections, of whatever nature, in structures of abnormal powers of resistance. Conversion into fibrous tissue by no means indicates that such accumulations will be permanent. It is quite probable that when this dense abnormal tissue is not subjected to constant physiologic strain it undergoes some form of degeneration leading to ultimate absorption as seen for example in certain types of pancreatitis and intra-abdominal adhesions. The best possible physical condition and

Roentgen rays are the only methods that have been supposed to aid this transformation.

Supplementary Treatment.

It has been so well established that patients may be apparently well and remain so even up to five years, only to succumb to an acute, explosive recrudescence, that there is positive indication for prolonged after-treatment, periodically repeated. The exceeding importance of protecting the general health cannot be overstated. Periods of even slight mental and physical depression have preceded, if they have not introduced, recrudescences. Except in two cases, in which the blood picture returned to normal and has remained there, we have not seen a single instance in which this evidence of a persistence of the infection was eliminated. Routine blood examinations are made as the patients report for observation, either at stated intervals of a few months or whenever untoward symptoms are suspected. The blood count has been found to give the earliest and surest evidence of an impending recrudescence or of the persistence of some focus of infection that had escaped detection. Apparently, recurrences of the disease, if taken very early, may be controlled by appropriate measures, whereas, if the infection increases to any extent in virulence the characteristic rapid fatal progress is to be expected.

Reexcisions.

Glandular recurrences have shown two types. One that follows soon after incomplete excision is of the softer, more cellular and acute variety. The other appears at a longer interval, perhaps more than a year after complete excision and repeated courses of the Roentgen ray, and is more chronic in every way, yet reexcisions usually done under local anesthesia have produced improvement, local and general, otherwise unobtainable. Removal of early recurrences is at times justifiable, but seldom is to be expected to help materially and has led to the opinion expressed by Fischer that reexcisions are always unwarranted.

During the period of quiescence, when there is no longer any ex-

ternal evidence of the disease, should be the time, hypothetically at least, when the administration of vaccine virtually prophylactically might be expected to be of the greatest benefit. Under such conditions the only judgment as to immediate improvement would have to be based on blood changes, and as the vaccine itself affects the blood picture nothing definite can as yet be stated. There has been no improvement noted in the return toward normal; either there has been no change or there was evidence of some degree of unfavorable variation.

One must constantly bear in mind that the safety of these patients lies in unremitting watchfulness and attention to details. The fact that otherwise there must be a certain fatality should lead to persistence to find the therapeutic measures indicated and give one courage to put them to practice.

RESULTS.

A cure or, better, a recovery can be considered as established only when there is no trace of the disease, including a normal blood picture, at least five years after the last manifestation of the infection. Recrudescence has occurred after a shorter period of apparent freedom. A case recorded by Eve,³⁰ had an interval of six years between a primary operation and the first recurrence but the diagnosis was not established.

Report of Cases.

These cases have been selected because two of them will show that, when the disease is taken sufficiently early, recovery, apparently permanent, can follow the simplest possible treatment, and the others will substantiate clinically and pathologically every contention that has been advanced, and may help to give impetus to what is hoped may be a beginning of more rational and more effective treatment of a disease that is temporarily of increasing clinical importance.

Case 1.—W. R., a boy aged 8, was seen in October, 1908. The duration of his illness was one year, cause unknown. There had been little effect on the general

30. Eve: Brit. Med. Jour., 1897, i, 584.

condition. The left side of the neck was almost completely filled with masses of fairly discrete glands, hard and not adherent to the deep structures or to the skin. It was a chronic type of the disease and apparently in a regressive phase.

Oct. 8, 1908, at Columbia Hospital a fairly complete excision in the left cervical region was done. The sternomastoid muscle and the internal jugular vein were not extirpated. The thoracic duct was ligated. Sections showed Hodgkin's disease positive histologically. The cultures were negative. (This was before the organism had been isolated.)

Recovery was prompt with smooth healing. Roentgen-ray treatments were begun early and continued to the limit of skin tolerance.

On April 1, 1909, the first blood count was made and showed the characteristic early picture.

July 24, 1909, the patient had begun to look and feel badly. He had had no recent roentgenotherapy. Recurrence was found in the region of the submaxillary gland. Other similar enlargements had appeared previously but had disappeared promptly under the use of the Roentgen ray. This treatment was therefore begun again.

Aug. 15, 1909, at Columbia Hospital the persistent recurrent growth was excised. Sections again were histologically positive. No cultures were made. Roentgen-ray treatment was instituted.

Jan. 22, 1910, the lungs and mediastinum were found negative as was also the left cervical region. A few small, superficial glands were found enlarged in the axillae and groins but nothing of pronounced significance. The blood picture was still that of the early stage.

April 12, 1910, the blood picture showed improvement.

June 21, 1910, the general condition was excellent. No evidence of recurrence was found.

Oct. 8, 1910, the von Pirquet test was negative. The blood picture showed continued improvement.

Oct. 22, 1910, the patient's health was good. Slightly enlarged glands were present in the upper angle of the left posterior triangle and near the hyoid bone. Examination otherwise showed negative results.

Feb. 13, 1912, the enlarged glands still persisted unchanged. The patient's general health was good, the blood picture favorable.

June 12, 1912, the patient's condition was unchanged.

March 2, 1913, the general condition of the patient was good, the liver and spleen not enlarged. The enlarged glands previously noted had slightly increased in size.

March 10, 1913, the blood picture was still that of the early stage but there was a marked increase in transitionals, 13 per cent., evidencing a less favorable condition.

March 18, 1913, at Columbia Hospital an excision of submaxillary glands was made also of those from the region of the mastoid and the hyoid bone. The glands, macroscopically were similar. Histologically, the sections showed nothing positively indicative of Hodgkin's. No cultures were made.

April 3, 1913, the patient was given 5,000 units of diphtheria antitoxin. No result was seen except urticaria.

Sept. 9, 1913, the records show the boy gaining in weight, the blood count nearly normal.

Jan. 27, 1914, the boy while thin, looked well and was growing rapidly. Physical examination revealed nothing significant, but the blood count unexpectedly indicated some recrudescence. The transitionals again increased to 11 per cent. A roentgenogram (made by Dr. Eisen) of the chest showed large glands in the mediastinum behind and below the left sterno clavicular joint. Roentgen-ray treatments were again begun.

Feb. 6, 1914, a polyvalent stock vaccine (Rosenow) was given subcutaneously.

April 4, 1914, the patient had received to date 62 billion organisms, no local or general reactions being noted. A roentgenogram (by Dr. Smith) showed numerous small bronchial glands to be enlarged. In the blood the transitionals were still high.

This boy was apparently a most unpromising patient. He was illnourished, wilful, inherently opposed to hygienic living and contemptuous of parental authority, but consistently cheerful and a fighter. Had the first operation been done as thoroughly as it now would be he would have been spared the recurrences. Even thus it is the only recurrence to be recorded after an approximately complete excision. His life has probably been prolonged materially, his distress has certainly been reduced to a negligible minimum. At present, six and one-half years after the onset and five and one-half years after the treatment was begun his condition warrants hope for an ultimate recovery. It is noteworthy, that, as the event proved, roentgenograms of the thorax alone demonstrated any pathologic adenopathy and the specificity thereof was determinable only by the blood picture.

Case 2.—In October, 1908, K. F. B., a man aged 22, had noted, six months before, slight but persistent irritation in the throat and nose. The patient lacked energy, and more recently there were enlarged glands in both sides of the neck and both axillae. Enlarged cervical glands were found chiefly in the posterior triangles. The axillary enlargements were within normal limits. The chest, abdomen and groins were negative.

Oct. 30, 1908, at Columbia Hospital, test excisions of cervical glands showed, microscopically, the early lesions of Hodgkin's disease. Tonsillectomy was done by Dr. Hitz, followed by Roentgen-ray and hygienic treatment.

Nov. 30, 1908, blood examination showed the early picture. It was in the study of the blood from this patient that the specificity of the blood changes were first determined. No other treatment was given than that indicated above.

Nov. 16, 1910, the blood examination was positive for the last time.

Dec. 15, 1912, the patient appeared in excellent health. A few small glands were present in both posterior triangles, the examination being otherwise negative.

Jan. 15, 1914, the patient reported himself perfectly well. The blood was negative. This case, though very early, was positively identified hematologically and histologically and proves that under prompt treatment recovery can occur.

Case 3.—In September, 1909, G. R., a boy aged 8, had, as was noted by a public-school nurse, shown enlarged glands eight months before, no cause being known and no effect on the general health being noted. A large mass of glands

had developed below the angle of the left jaw. No other glandular enlargement was found. The chest and abdomen were negative.

Sept. 30, 1909, a test gland was excised and showed positive Hodgkin's changes.

Nov. 1, 1909, the blood examination was positive, the early blood picture being present.

Nov. 15, 1909, at Columbia Hospital, the enlarged glands were excised but by an incomplete operation. Tonsillectomy (Dr. Hitz) was done, the enlarged glands showing marked periadenitis. Sections showed positive Hodgkin's disease. The large and small glands were involved in the typical average type of lesion. Cultures were negative; it was through this and other failures that the proper technic was finally developed. Roentgen-ray and hygienic supplementary treatments were instituted.

Oct. 30, 1909, blood examination was positive, but continued better.

Sept. 7, 1910, recurrence was noted beneath the angle of the jaw (local recurrence following incomplete operation). Excision was done and sections later were found to be positive histologically. The blood was positive, the early picture present.

Oct. 8, 1910, the von Pirquet test was negative.

Dec. 25, 1910, the general condition was excellent, no recurrence being recognized. Chest and abdomen were negative.

Feb. 9, 1911, the general health of the patient was good. Small glands were noted in both sides of the neck and in both axillae but within physiological limits.

March 7, 1911, the patient was readmitted to the hospital because of profuse bleeding from the nose. The coagulation time was sixty-seven minutes. Epistaxis soon ceased and the patient was discharged.

April 17, 1911, the patient was again admitted to the hospital bleeding now from both nose and gums. It was noted for the first time that there were fever, a distended abdomen and an enlarged spleen. The boy was extremely anemic. The platelets were virtually absent. The bleeding continued. Blood transfusion was twice repeated, the first time with, the second without benefit. A 0.3 gm. dose of salvarsan was given intramuscularly and was followed by some reduction in temperature.

April 20, 1911, a first attack of relapsing fever of the Murchison (usually misnamed Pel-Ebstein) type began.

June 15, 1911, there was a recurrence of fever. Salvarsan, 0.3 gm. was administered intravenously after which there was a reduction of fever for two days, also a decrease in the size of spleen and liver, which had recently become palpable.

June 22, 1911, another febrile paroxysm began. The spleen was not affected, the liver, perhaps, a little smaller.

Sept. 1, 1911, a mass of recurrent glands in the region of the submaxillary was excised and found positive, histologically, for Hodgkin's disease. The main mass of gland tissue was emulsified and injected into guinea-pigs but without result. The cultures were contaminated.

Sept. 20, 1911, there was recurrence of fever and bleeding from the gums and nose. The fever continued for some time. There was a rapid local recurrence of glands. Salvarsan, 0.2 gm. was given intravenously without effect. Increas-

ing emaciation and abdominal distention continued until death occurred from starvation.

Jan. 22, 1912, postmortem examination of the abdomen disclosed chylous ascites, advanced retroperitoneal glandular and splenic involvement. Cultures contaminated.

This case shows the futility of incomplete operations. The disease was of the fairly early chronic type, and, possibly, in spite of the periadenitis, under more radical and vigorous treatment should have done as well at least as the patient in Case 1, alive after six years.

Case 4.—December, 1909, F. v. S., a boy, aged 10, had been lacking recently in his usual energy and appeared to be undernourished. Slightly enlarged glands were present in both sides of the neck in both axillae and both groins. Expiration at the left apex was harsh and prolonged but no râles were heard. The blood was positive, showing the early picture of Hodgkin's disease.

Jan. 10, 1910, tonsillectomy (Dr. Hitz) was done at Columbia Hospital and test glands were removed from neck, axilla and groin. The cervical gland was positive histologically. The axillary and inguinal glands were possibly (?) suggestive. The treatment was hygienic and the Roentgen ray was used.

April 26, 1910, a slight general glandular enlargement still existed. The patient was growing rapidly but was gaining in weight and strength.

Oct. 5, 1910, the condition was quite the same. The von Pirquet test was positive.

Sept. 10, 1913, the glands were still slightly enlarged. Harsh breath sounds were heard at the left apex and the von Pirquet test was positive.

Jan. 6, 1914, the general condition was excellent, the glands quiescent and the blood picture was normal. The conditions at present are the same. Except for the positive histologic diagnosis this case might be open to serious question. The absence of symptoms and persistence of a normal blood picture for four years make this almost certainly a recovery and indicate again the efficacy of the simplest treatment when given early.

Case 5.—In October, 1911, A. F., a woman, aged 33, was seen. She had begun to run down two years ago. Five months later she first noticed periodic attacks of nagging pain in the region of her spleen which lasted a few days and recurred every few weeks. About fifteen months ago the sense of lassitude was still more increased but was notably worse at intervals. One year ago she had protracted subacute conjunctivitis and rhinitis and a month or two later noted the first glandular swelling. Lassitude and anemia increased. Glands appeared high in the right anterior cervical triangle and then spread. Eight months ago, after an acute cold, she noted enlarged glands in the left side of the neck. She would feel "grippy" at intervals and noted changes in the glands at such times. Five months ago enlargements appeared in the right axilla. She had been having some medical attention and knew that her temperature had been as high as 102 F. during the periods of exacerbation. This history alone indicates a most unfavorable case.

Oct. 9, 1911, the glands in both triangles and in both sides of the neck were enlarged, but were fairly discrete. The greatest cervical involvement was beneath the right mastoid muscle at the level of the angle of the jaw. Pathologic

enlargements were also present in the right axilla. The chest and abdomen were negative.

Oct. 10, 1911, the von Pirquet and Wassermann tests were both negative.

Oct. 16, 1911, salvarsan, 0.4 gm. was given intravenously. It was thought to be indicated because of her regular elevation of temperature and apparently had some lasting effect if it was not followed by a normal period of remission.

Oct. 23, 1911, at Columbia Hospital, the right tibia was explored because of signs of acute osteomyelitis. Edema was present between the skin and the periosteum. Nothing else was identified macroscopically or microscopically. A test gland excised from the right axilla was positive histologically.

Nov. 10, 1911, Roentgen-ray treatment of the left cervical and right axillary glands was begun.

Dec. 11, 1911, at Columbia Hospital tonsillectomy was done by Dr. Hitz. After the Roentgen-ray treatment a gland was excised from the axilla for comparison. The gland showed positive Hodgkin's disease and some increased sclerosis, but no cellular destruction.

Feb. 12, 1912, the glands in the left neck were smaller. At Columbia Hospital an extensive but incomplete excision of glands was made from the right neck. The glands were soft and showed extensive periadenitis and positive Hodgkin's disease. Cultures showed *B. hodgkini*, pure. This we believe was the first time that the organism was ever cultivated.

May 1, 1912, a few glands were found in the left neck, one in the right. The axillae were free.

June 3, 1912, 5,000 units of diphtheria antitoxin were given without any evident effect.

July 20, 1912, both sides of neck and both axillae were negative. There was no more tendency toward rise of temperature.

Jan. 29, 1913, the glands were reexcised from both axillae because of recurrence with manifest effect on the general condition. The glands were positive but of less acute type; cultures were of *B. hodgkini* pure. Later a control gland was excised from the right axilla but as Roentgen-ray treatments had been afterward given this time no growth resulted from the gland.

March 18, 1913, recurrences were excised from the upper right anterior triangle, followed by iodine disinfection. The gland process was less acute but was histologically positive. Cultures were not taken.

March 5, 1914, a small recurrence was excised from the right axilla. Otherwise the physical examination was negative.

April 1, 1914, a roentgenogram of the chest made by Dr. Smith was negative.

May 6, 1914, as the blood picture was still positive, subcutaneous autogenous vaccination was tried and about 500 million were given during April. At the same time recrudescence was developing in the right axilla, wherefore vaccination was stopped.

May 11, 1914, a complete radical dissection was made in the right axilla which was disinfected with iodine and drained. The glands were more fibrous histologically, the cultures were of *B. hodgkini*.

The hospital records in this case were lost in part, and the preceding summary, while not absolutely accurate, is virtually correct. It indisputably illus-

trates from a therapeutic point of view the folly of incomplete excisions and indicates the dangers to which an individual is thereby subjected. This patient was the first from whom the *B. hodgkini* was obtained and it was grown from recurrences three times subsequently and always in pure culture, except the one time after treatment of a control gland by the Roentgen ray. The development of axillary recurrences during the period when vaccine was being given is suggestive. In this case, possibly of five years' duration, treatment was begun in the second stage after febrile manifestation had appeared, giving a very unfavorable prognosis. Again we believe that life has been prolonged, discomfort relieved and that there is a possibility of ultimate recovery. It is such experiences as this that give one the encouragement to attack radically the less favorable cases.

Case 6.—In December, 1913, P. M., a man, aged 27, was referred by Dr. John I. Specht. The disease had been in progress about eight months, and began with pain in the upper left chest with loss of weight. Three months later sleeplessness and night sweats developed. Four months ago the patient noticed pea-sized tender glands under the skin of the chest over the manubrium sterni. The gland next noticed to have become enlarged was above the left clavicle and was followed by two or three others near by. These soon fused, grew rapidly and became fixed. Pains in the upper right chest were next noticed just lateral to the sternum. Extension successively occurred to the right axilla, right neck and left neck. For two months obstructive dyspnea had been present, while cough had existed since the onset. The principal distress had arisen through attacks of orthopnea and sleeplessness. The neck involvement was sufficient to restrict head movements and the arms could not be adducted because of axillary masses. Edema of the skin of the face, neck and chest down to the nipples was observed. The liver and spleen were not enlarged. Marked intrathoracic involvement was present especially on the left side.

Oct. 22, 1913, at Columbia Hospital, tissue was excised for culture. The blood picture was positive. The glands showed lesions of acute Hodgkin's with focal necroses. The cultures gave a characteristic diphtheroid, pure. Grown with the greatest difficulty it was probably the most virulent culture secured. The patient was given massive doses of the Roentgen ray without regard to danger of burning and subjective relief was marked. Objectively, little if any change could be recognized.

Jan. 12, 1914, the hypodermic administration of both autogenous vaccine and diphtheria antitoxin was begun.

Feb. 9, 1914, the blood examination showed that a transition to a late stage had occurred. Subjectively the patient seemed better after antitoxin and the Roentgen ray. No effect was evident from the vaccine.

Feb. 22, 1914, the leukocyte count was 72,500, lymphocytosis 69 per cent. This change lasted less than a week, when the count returned to that of Feb. 9, 1914.

March 10, 1914, the patient had become progressively weaker. Very short periods of remission were shown in the general condition and in the temperature. He received 2,000,000,000 autogenous vaccine subcutaneously and 218,000 units of antitoxin serum (Memorial Institute), 33,000 of which were given intravenously, without manifest improvement. He died quietly and suddenly while

comparatively comfortable. At necropsy the pectoral muscles, intercostal muscles, parietal and visceral pleura and parietal pericardium were riddled with nodules. Involvement of bronchial, mediastinal and retroperitoneal glands, and of lung, liver and spleen was found. There was general miliary tuberculosis and probably primary mediastinal disease.

Case 7.—In January, 1914, E. G., a young lady, aged 18, was referred by the Mayo clinic. No etiologic factor was disclosed. The gland first noted was in the left posterior triangle, possibly six months before. Within two months extension to the left axilla had been noted. Within the last few weeks enlargements had been noted in the right neck and right axilla. There had been no effect on the general health save dyspnea on exertion.

Jan. 15, 1914, in the left neck the glands in both triangles were much enlarged and firmly fused. The posterior triangle was filled with a fixed mass up to the level of the larynx. The overlying skin was edematous. In the right neck a chain of glands was palpable in the posterior triangle. The left axilla was filled from apex to base with a fused mass. Complete adduction of the arm was impossible. In the right axilla there was a less but a distinct involvement. The groins and abdomen were negative, the liver and spleen not enlarged. The whole left upper front of the thorax was involved, the skin above the left breast showing a brawny thickening. A roentgenogram showed both mediastinums filled with large glands.

Jan. 19, 1914, test glands excised were both positive, the cultures giving *B. hodgkini* pure.

Jan. 31, 1914, tonsillectomy was done by Dr. Hitz. The tonsils were small. From the left a small amount of milky fluid was expressed. Histologically it showed disease.

Feb. 23, 1914, under maximum doses of the Roentgen ray some diminution of the visible glands was observed. A roentgenogram by Dr. Smith showed increased mediastinal involvement.

Feb. 26, 1914, at Columbia Hospital, a complete operation was done on the left neck with excision of the sternomastoid muscle and jugular vein. Extreme fibrous periadenitis was encountered. The thoracic duct was opened. Closure by suture was impossible but it later responded to packing. A fairly complete excision of the glands of the left axilla was made.

March 16, 1914, no evident effect had been observed since Feb. 5, 1914, of 400,000 autogenous vaccine which were given subcutaneously without reaction and 30,000 units of diphtheria antitoxin without evident effect.

March 21, 1914, both sides of the neck were negative as was also the right axilla. The left showed two small glands. The patient returned home where her physician, Dr. G. A. Lott, gave 10,000,000 autogenous vaccine subcutaneously up to May 14.

May 18, 1914, the patient had gained in weight and strength and asserts that she never felt better. A slight dyspnea still persists. Deep in the anterior, inferior angle of the posterior triangle of the right neck a few enlarged glands were noted as following a recent right-side parotitis. On the left side induration and pigmentation from the Roentgen ray persists. No glands were palpable. Low in the right axilla a freely movable bantam-egg-sized mass was the only

palpable involvement. In the left axilla the scar was soft and freely movable. There was no further involvement; the abdomen was negative.

May 22, 1914, the von Pirquet test was negative. The blood showed no change over the previous count. A gland from the lower right axilla was excised under local anesthesia and gave positive Hodgkin's. Cultures were not made.

June 4, 1914, the entire examination except for glands low in the right posterior triangle was negative externally. The abdomen was also negative, the liver and spleen not enlarged. The chest involvement was apparently unchanged. The blood since April 4 had been indicative of late changes. This case illustrated the resistance of intrathoracic involvement to treatment and the unfavorable prognosis indicated by the periadenitis. If it be possible to prolong this life and more particularly to reduce discomfort, mental as well as physical, the maximum benefit under the conditions will have been obtained.

A RAPID AND SIMPLE METHOD OF TESTING DONORS FOR TRANSFUSION.

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The increasing popularity of transfusion as a therapeutic measure has brought with it the need of a test whereby the suitability of donors may be rapidly determined. There are already many instances on record of serious symptoms or death following the rapid breaking down of blood after transfusion. The evidence is convincing that such instances may be avoided if a donor is selected beforehand by means of certain simple tests carried out in vitro. Unfortunately these, as performed at present, require time and labor. It is with the aim of providing a test rapid and simple enough to be employed even in emergencies that the following method has been devised:

Method.

Collection of the Blood.—The blood is taken from the patient and the prospective donors in a 1:10 mixing pipet, such as is used in counting leukocytes. The pipet is rinsed beforehand with 10 per cent. sodium citrate in water; the citrate solution is drawn up to the mark 1; the pipet is rapidly filled with blood from a puncture of the ear or finger; and without pause the mixture is expelled into a small, narrow test tube. There is thus obtained a citrated blood containing slightly less than 1 per cent. of citrate. The pipets which we have employed hold only 0.25 c.c. of fluid. This much blood is easily obtained from a single puncture. There is no objection to increasing the flow by pressure. Should it cease before the pipet is full, the blood must be at once expelled into a test tube, in order that it may mix with the citrate and clotting be avoided. The mixture is then taken up again, a new puncture made, and the pipet completely filled. After each blood is obtained, the pipet is rinsed with citrate, then with distilled water, then with fresh citrate, and it is ready for another blood. If several donors are to be tested, two pipetfuls of citrated blood should be obtained from the patient. It is best to take them from different puncture wounds, in order to avoid a possible clotting in the pipet.

Mixing.—The mixing is done in pipets with a capillary end—the so-called Wright pipets obtained by drawing out glass tubing in the flame. The citrated

bloods are used as such, and two combinations are made of the patient's blood with that of each prospective donor—a mixture containing nine parts of the patient's blood to one of the donor's, and a mixture of equal parts of the two. The proportions used need be only approximate. In case of emergency the first of the mixtures will suffice, since by its use the most dangerous possibility, namely, that the blood of the recipient may destroy that of the donor, can be ruled out. Following the technic usual with Wright pipets, the capillary tube is marked, blood is drawn to the mark, and each column of the blood is separated with an air bubble from the next that is drawn up. To insure proper mingling, each mixture should be expelled on a slide, or Widal plate, and then drawn high in the pipet, which may be sealed off in the flame in case the examination is not to be made for some time.

Incubation.—No incubation in the ordinary sense is necessary. The pipets are kept at room temperature, and readings are begun after two minutes, if there is need to hurry. The readings are for agglutination, and even within two minutes this is plainly evident, except when the agglutinating forces are notably weak. In the final choice of a donor it is safest to rely on results obtained after the mixtures have stood for fifteen minutes. But the ruling out of individuals with unfit blood may be begun practically at once.

Readings.—The capillary end of each pipet is broken, a small drop of the blood expressed on a slide, a large drop of normal salt solution superimposed without mixing, a coverslip put on, and the preparation examined for agglutination under the microscope. Fresh preparations can be made at intervals if desired. The salt solution is not absolutely necessary; but very clear pictures are obtained as the blood spreads in it. When agglutination has occurred, the red cells show a characteristic clumping, sometimes in small masses, often in large ones that are very evident macroscopically. The clumps in each preparation are fairly uniform in size. The picture is absolutely different from that in mixtures of nonagglutinating bloods under similar conditions. In these, the cells lie free or in rouleaux, just as in a single blood. But in agglutinating mixtures the cells are stuck together "every which way," and, where the film is thin, they do not separate but lie connected with one another in irregular heaps. If pressure is put on the coverslip, a very characteristic phenomenon may sometimes be seen. The agglutinated cells pull out in strands as though they consisted of some sticky substance. The most striking pictures are encountered when there are nine parts of an agglutinating blood to one that is agglutinated. Here large discrete masses lie scattered amid unclumped red cells.

If there is no clumping in the preparations made after the mixtures have stood fifteen minutes, the assumption is warranted that the bloods do not agglutinate or hemolyze each other. The experience of previous workers has taught that in such instances transfusion is safe. But if clumping is present in the 9:1 mixture and to a less degree or not at all in the 1:1 mixture, it is certain that the blood of the patient agglutinates that of the donor and may perhaps hemolyze it. Transfusion in such cases is dangerous. Clumping in the 1:1 mixture with little or none in the 9:1 indicates that the plasma of the prospective donor agglutinates the cells of the prospective recipient. The risk from transfusing is much less under such circumstances, but it may be doubted whether the blood is as useful as one which does not and is not agglutinated. A blood of the latter kind should always be chosen if possible.

For practical purposes these findings suffice. But if there is a desire to know whether *both* bloods contain agglutinins, a 1:9 mixture should be made. If this and the 9:1 mixture show large clumps, whereas the clumps are smaller when the bloods are mixed in equal parts, two agglutinins must be present.¹ Should there be only one agglutinin, little clumping or none will be observed when the blood containing the agglutinin is diluted with nine parts of the other blood.

Difficulties.—The single technical difficulty of the method is that of clotting, and to avoid it the blood should be taken as rapidly as possible. With normal blood, trouble is seldom experienced, but in pathologic instances a thin web may form in the test tube into which the blood is expelled. The clotting begins in the calibrated tube of the pipet which, as the blood is taken, is swept free of citrate; and the use of a strong citrate (15 per cent.) will not prevent it. But the clot is always thin and may be picked out of the blood and the latter used for the tests. In the pipet even small clots are troublesome, since they form a locus for new ones when another blood is taken. They are best dissolved out with 5 per cent. potassium hydroxid.

The likelihood that something else may be mistaken for agglutination is practically nil. The presence of fibrin strands and clumps of platelets and white cells absolutely differentiates bits of clot. Dried blood will not be found except in case of carelessness, and it is unlikely to cause confusion. Its nearly homogeneous, cheesy appearance under the microscope is sufficiently distinctive.

Comment.

The test here described has some features in common with two of the methods already in use. Therefore, to point out wherein it is an advance over these will not seem amiss. Epstein and Ottenberg² use Wright's pipets, collecting blood in them for serum, and making the ultimate test mixtures in them. But the mixtures consist of serum and washed red cells; they are incubated for two hours, and the readings are macroscopic. Weil³ employs citrated bloods, making, in test tubes, three mixtures of the same relative proportions as ours. Two c.c. of each blood are required, thus making necessary aspiration from a vein; the tubes are incubated for an hour, and the readings are macroscopic. By means of the test of Epstein and Ottenberg, aspiration from a vein is avoided; and by Weil's method

1. This phenomenon has been described by Ottenberg in an interesting study of the conditions under which human bloods show agglutination. He used mixtures of defibrinated whole blood. (Ottenberg, R.: Jour. Exper. Med., 1911, xiii, 425.)

2. Epstein, A. A., and Ottenberg, R.: A Method for Hemolysis and Agglutination Tests, Arch. Int. Med., May, 1909, p. 286.

3. Weil, Richard: Sodium Citrate in the Transfusion of Blood, THE JOURNAL A. M. A., Jan. 30, 1915, p. 425.

the need to separate serum, wash the red cells and make reciprocal observations is done away with. In both instances, however, the time and the labor of testing donors is very considerable. Using our method, it is only necessary to obtain citrated bloods from a finger-prick, make two mixtures of them in capillary pipets, and, by reading with the microscope, the test is finished within a few minutes.

The method involves several important assumptions. They may be summed up in two questions: 1. Is the presence or absence of agglutination an index to the injurious qualities of a blood? 2. Are the microscopic findings with mingled, citrated bloods that have stood fifteen minutes at room temperature as certain in their indication as macroscopic findings after longer periods with mixtures of serum and 5 per cent. washed red cells—that is to say, as certain as the test most used at present?

The first question can be answered from the literature. Leaving from consideration disease in the donor, two injurious influences must be thought of when a foreign blood is introduced into the human body, namely, hemagglutination and hemolysis. The latter is far the more serious. Moss⁴ has found in extensive observations that agglutination frequently occurs without hemolysis, but that hemolysis is always associated with or preceded by agglutination. This conclusion he has substantiated with seventy-five successful transfusions⁵ in which the donors were selected by means of the agglutination test. It is well known that human beings fall into four groups as regards the agglutinins in their serum and the capacity of their corpuscles to be agglutinated. Moss was careful to select for transfusion individuals of the same group. Without going deeply into the matter we may say that, when serum and washed cells are used, reciprocal observations are necessary to determine whether two individuals belong to the same group. But in mixtures of whole citrated bloods, agglutination is only absent when this is the case.

In our test, agglutination is looked for with the microscope. This

4. Moss, W. L.: Johns Hopkins Hosp. Bull., 1910, xxi.

5. Moss, W. L.: Am. Jour. Med. Sc., 1914, cxlvii, 698. See also, for the bearing of agglutination on transfusion, Ottenberg, Reuben, and Kaliski, D. J.: Accidents in Transfusion, THE JOURNAL A. M. A., Dec. 13, 1913, p. 2138.

does not mean that macroscopic readings are impossible, for when clumping is at all outspoken in the slide preparation, it is plainly evident to the naked eye. Observations can, in emergency, be made in this way. But with the microscope, as one would naturally suppose, the readings are more sensitive and more precise. When antirabbit goat serum is mixed with rabbit red cells, clumping is observable microscopically in dilutions of serum much higher than those in which it is evident macroscopically in the test tube. This can be noted also with dilutions of an agglutinating human serum mixed with human red cells and with undiluted human serums weak in agglutinins. An agglutinative clumping is rarely doubtful microscopically, for the cohesion of the cells, even when only two or three are concerned in each clump, is absolutely different from rouleaux formation. In the test tube an agglutination is often difficult to distinguish from sedimentation; and the shaking which throws up the clumps into the fluid that they can be viewed may break them so that they do not reform.

Our experience is that if agglutination occurs at all it will be noticeable microscopically within five minutes. But it is better to let the mixtures stand longer. We have no hesitation in saying that agglutination can be told with the microscope as certainly after fifteen minutes as in two hours with the old macroscopic method. The explanation of this is probably simple. The clumps which become visible in the test tube after an hour or more are in general not the primary clumps of an agglutination, but the result of the massing together of many such clumps, which themselves are often small. Such secondary massing requires time, whereas the primary clumps form almost immediately.

That agglutination is sometimes better at room than at body temperature is well known.⁶ Repeated trial has shown that the incubation of our agglutinating mixtures seldom increases the rapidity of the reaction. In some instances agglutination was much more pronounced after fifteen minutes at room temperature than in two hours at body heat. An altered distribution of the agglutinin may account for this.

6. Paltauf, R., in *Kolle-Wassermann's Handbuch der pathogenen Mikroorganismen*, ii.

It has been repeatedly shown that an excess of red cells may mask the presence of an agglutinin or hemolysin, and that hemolysis is much influenced by the presence of neutral serum. Agglutination is practically independent of this latter factor.⁷ The influence of small amounts of sodium citrate does not seem to have been determined. The following is one of several experiments dealing with these points in their relationship to our test:

Protocol of Experiment.

By venous puncture some cubic centimeters of a human blood, *A*, were taken with citrate (1 part 10 per cent. citrate to 10 parts blood) and some obtained for serum. Graded dilutions of the serum were made with salt solution, and mixed in small test tubes, as also in Wright's pipets, with equal parts of 5 per cent. suspensions in salt solution of certain washed red cells, *B* and *C*, which the undiluted serum agglutinated.

Dilutions of the plasma of the citrated blood *A* were made, similar to those of the serum, but the proportion of red cells was kept the same as in the original citrated blood. For this the citrated blood was distributed in a number of graduated tubes, centrifugalized rapidly, the supernatant fluid measured and diluted appropriately, and, when the red cells had been suspended and centrifugalized, all of the fluid was pipetted away except the original volume, and the cells were resuspended. The diluent used was sodium citrate in salt solution (1 part 10 per cent. sodium citrate to 9 parts 0.95 sodium chlorid). Nine parts of each of the combinations thus obtained were mixed in Wright pipets with one part of the whole citrated bloods *B* and *C*. The microscopic findings after fifteen minutes were compared with those in the serum mixtures after one and two hours. They gave identical results. Agglutination occurred when dilutions of serum or plasma up to 1 part to 7 of the diluent had been used for the mixtures, but not when 1 part to 15 had been employed. It was slight with 1:7 plasma and no better marked with 1:7 serum.

The experiment shows that a weak agglutinin may be demonstrated quite as well with mixtures of citrated whole bloods as with serum and a diluted suspension of washed red cells in salt solution. The interference of neutral red cells does not constitute a difficulty.

CONCLUSION.

The test here described compares well in delicacy with those already in use, but we wish to insist only on its practicability. It enables one to determine within a few minutes, so far as agglutination and hemolysis are concerned, whether or not the blood of a donor is suitable for transfusion.

7. Hektoen, Moss, Ottenberg.

THE LYMPHOCYTE IN NATURAL AND INDUCED RESISTANCE TO TRANSPLANTED CANCER.

II. STUDIES IN LYMPHOID ACTIVITY.

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(Received for publication, May 28, 1915.)

In former communications, we have shown that the resistance to heteroplastic tissue grafts apparently depends on the activity of the lymphocyte. The chick embryo, which we found¹ to be devoid of any resisting power to the growth of grafted tissues from a foreign species, could be rendered as refractory as the adult animal, by the introduction of a bit of adult homologous tissue rich in lymphoid elements, such as spleen or bone marrow.² Furthermore, an adult animal deprived of the major portion of its lymphoid system by means of repeated small doses of x-ray, no longer resists the growth of heterologous tissues. The tissue cells from a foreign species will grow actively till such a time as the depleted lymphoid system of the animal is well advanced in regeneration.³

Histologically, there is a striking resemblance between the appearances presented by the failing heteroplastic graft in the foreign host and the cancer graft in the so called immune animal. The phenomenon is the same, whether the state of resistance to the cancer is one possessed naturally by the animal, a condition termed natural immunity, or whether it is of the nature of induced immunity developed by previous treatment of the animal. The details of the various stages of the processes as observed under the microscope need not be gone into here.⁴ It is only necessary to say that the

¹ Murphy, Jas. B., *Jour. Exper. Med.*, 1913, xvii, 482.

² Murphy, Jas. B., *ibid.*, 1914, xix, 513.

³ Murphy, Jas. B., *Jour. Am. Med. Assn.*, 1914, lxii, 1459.

⁴ For the literature see Da Fano, C., *Ztschr. f. Immunitätsforsch., Orig.*, 1910, v, 1.

constant and undisputed fact is that in both the heteroplastic tissue graft and the homologous cancer graft in an immune animal, there is a pronounced collection of small round cells at the edges of the grafts and densely infiltrating the tissues about.⁵ The lymphoid elements appear quite early in the process and last till it is completed. It is notable that these elements are absent about the growing cancer graft in the highly susceptible animal.

With the above fact at hand it would seem, arguing from analogy, that the lymphocytes might play an as equally important part in immunity to transplanted cancer as they appear to play in the resistance phenomenon to heteroplastic tissue grafts. With this problem in mind the following experiments were planned.

Resistance to Transplanted Cancer.

It is unnecessary to go into the details and theories of the various types of resistance to the transplanted cancer. This has been done at length in numerous communications on the subject.⁶ The essential facts are that mice may be rendered relatively immune for a period by giving a subcutaneous or intraperitoneal injection of a certain amount of homologous living tissues at least ten days before inoculating the cancer graft. Various tissues have served, such as hashed embryo, embryo skin, spleen, and red blood cells, for this purpose. The efficiency of the protection varies considerably, depending on the type and amount of tissue used, as well as on the growth power of the cancer inoculated. A certain proportion of mice inoculated with cancer show a natural refractory state which varies in different families of mice and to an extent according to the virulence of the tumor. Sometimes this natural resistance is so effective that the cancer graft does not become established, while in the less resistant animal the introduced tumor may grow for a time and only later be overcome and absorbed. These are the types of immunity which we have elected to study from the point of view of their general lymphoid reaction.

⁵ For general review and literature on the subject see Woglom, W. H., *The Study of Experimental Cancer, A Review, Studies in Cancer and Allied Subjects*, New York, 1913, i, 128.

⁶ Woglom, *loc. cit.*

In all we have made detailed studies of the blood in over eighty mice before and at intervals after inoculation with cancer in both immune and susceptible animals. Only one of these experiments will be reported as the others gave practically identical results.

Experiment I.—20 white mice of about the same age and size were selected. A white blood count and a differential count were made to establish the normal in each animal. 10 of the mice were given a subcutaneous injection of 0.3 cc. of defibrinated mouse blood. 10 days later all 20 animals were inoculated with equal sized grafts of a transplantable mammary carcinoma of the mouse.[†] Blood examinations were made after 24 hours and at intervals for something over 50 days. The grafts were measured and recorded weekly. The protection in the immunized animals of this series was 100 per cent. Among the controls, 60 per cent grew their grafts and 40 per cent were naturally immune. From the white count and differential, we have estimated the actual numbers of the various types of white cells present.

Typical examples of each group are shown in Text-fig. 1, A being an immunized animal, B a control animal with a growing tumor, and C a naturally immune animal. In these counts we have grouped together the large and small lymphocytes; and have not differentiated the types of polynuclear cells. The changes that have taken place have been, however, almost entirely in the small lymphocyte group. In Curve A, here given, no blood examinations were made between the primary count and one twenty-four hours after the cancer inoculation, but a study of a large series of animals has shown no variation in the count before the introduction of the cancer graft. It is assumed, therefore, in this case that the normal level is maintained.

The immediate increase in the circulating lymphocytes present in the immunized animal is a constant finding and in a large proportion of the cases has risen to 100 per cent or more above the normal level during the first twenty-four hours. This is well shown by the composite curve made from the average of all of this series (Text-fig. 2 A). In Curve C of Text-fig. 1 we show a typical example of a naturally immune animal. Here the lymphoid increase is delayed, and is preceded by a slight fall. The composite curve (Text-fig. 2 C), however, shows that as an average there is slight if any change during the first twenty-four hours, but the reaction develops

[†] This tumor was propagated by Dr. Woglom and kindly given us by him.

sometime during the first week and continues to rise with some variation to a level between 100 and 200 per cent above normal. These animals show, as a rule, some growth of the cancer during the first week but after this, retrogression sets in, continuing to complete absorption. In the susceptible animal (Text-fig. 1 B) there is a decrease in the lymphocytes and a tendency for the polynuclear cells to increase slightly. This particular animal offered little resistance apparently, as the rate of growth of the tumor, shown at the bottom of the text-figure in silhouette, would indicate. This is not uniformly so, however, as is demonstrated in the composite curve (Text-fig. 2 B) taken from a number of animals with growing tumors. Here is shown a slight reaction at the end of the first week which, however, subsides as the tumor increases in size. In one animal in the series, there was a fairly marked reaction of the lymphocytes in the second week, with a marked retardation in the growth of the tumor. The cancer later broke through the resistance which was accompanied by a corresponding drop in the mononuclear elements of the blood.

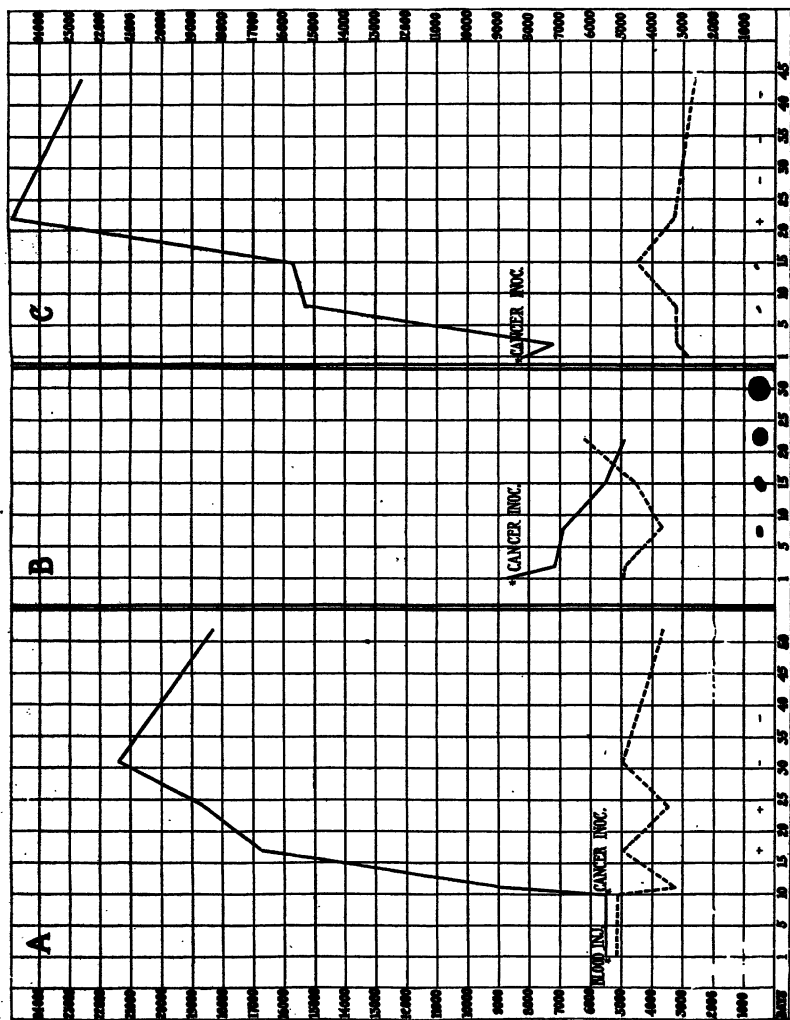
This series of experiments shows that the resistant state to transplanted cancer, whether of the natural or induced variety, is accompanied by a marked lymphocytosis. In the highly susceptible animals this phenomenon is absent, while in the less resistant ones, where the tumor is retarded but not checked absolutely, there is a slight but apparently inadequate reaction of these cells. Whether the immunity is dependent on the lymphoid increase or not is a different matter. If the reaction is a necessary part of the immunity process, the destruction of the lymphocytes in the immunized animals should be accompanied by a loss of the immunity.

The Effect of Lymphoid Destruction on the Induced Immunities to Transplanted Cancer.

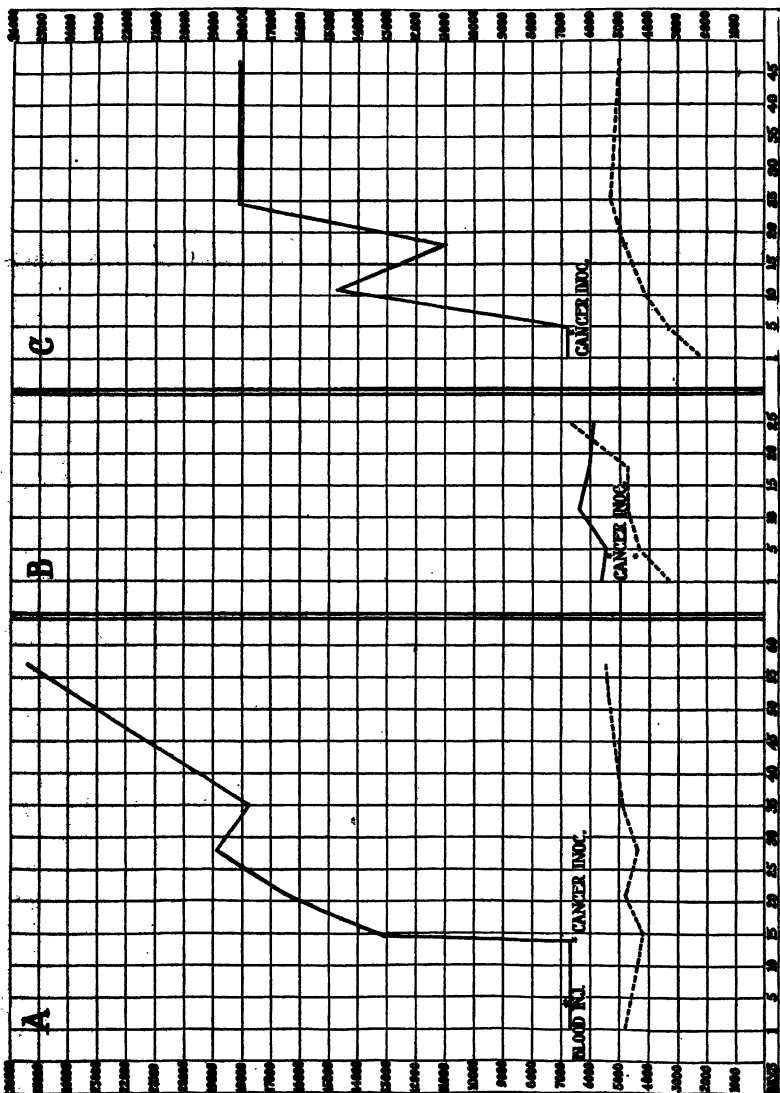
Heineke has shown that x-ray effect is manifest first in its destruction of the lymphocyte.⁸ We have found that repeated small doses of x-ray⁹ destroy the major portion of the lymphoid system of the mouse without causing apparent injury to other tissues or pro-

⁸ Heineke, H., *Mitt. a. d. Grenzgeb. d. Med. u. Chir.*, 1905, xiv, 21.

⁹ Murphy, Jas. B., and Ellis, A. W. M., *Jour. Exper. Med.*, 1914, xx, 397.



TEXT-Fig. 1. This shows the blood changes in typical examples of A, an animal with induced immunity; B, one with a growing cancer (shown in silhouette at bottom); and C, a naturally immune animal. The solid line represents the actual number of lymphocytes, and the dotted line the polymorphonuclear leucocytes. The first count in Curve A was made before the immunizing injection, and in the others before the cancer inoculation.



TEXT-Fig. 2. Composite curves formed by averaging the counts for all animals in each group. A, composite curve from a number of immunized animals. B, composite curve from a number of susceptible animals with growing cancer. C, composite curve from a number of animals, naturally immune to transplanted cancer. The solid line represents the actual number of lymphocytes, and the dotted line the polymorphonuclear leucocytes.

ducing detrimental effect to the general health of the animal. This method offers a means of testing out the importance of the lymphocytic reaction in the immune states to cancer. We have found it necessary to regulate the dose very carefully, as too large or too frequent doses will cause such a general disturbance in the metabolism that tumors after they develop will grow slowly. Likewise, too small an amount of x-ray will only partially bring about the desired effect. The following experiment is one of four which have given uniform results.

Experiment II.—30 adult mice of about the same age and size were selected. 20 of these were given 0.3 cc. of defibrinated mouse blood, and 10 were put aside for controls. 10 of the immunized animals were subjected to small doses of x-ray for 7 consecutive days by means of the Coolidge tube, 10 milliamperes, 3 inch spark gap, and exposure of 1 to 2 minutes. Blood examination showed this to be sufficient to reduce markedly the circulating lymphocyte, leaving the general health of the animal unaffected. 10 days after the blood injection, all 30 animals were inoculated with a fragment of a transplantable mouse cancer. Text-fig. 3 gives the rate of growth of cancer in the various groups. The x-rayed immunized group shows the same number of takes as does the normal series, but the tumors in the x-rayed animals grew more rapidly.

The experiment would seem to indicate that the destruction of the lymphocytes between the period of immunizing injection and cancer inoculation by preventing the usual lymphoid reaction, suffices to abolish the immunity which would otherwise be present.

The Effect of Lymphoid Destruction on Natural Immunity to Transplanted Cancer.

The above experiments have shown that the lymphoid reaction is the same in the naturally immune animals and in the animals with induced immunity. We would expect, therefore, that treatment of normal animals with x-ray before inoculation with cancer should greatly increase the number of takes.

Experiment III. Series A.—20 rats of the same age and size were selected. 10 of them were given 12 doses of x-ray on consecutive days in amounts sufficient to reduce greatly the lymphoid elements. All the 20 animals were then given grafts of the Jensen rat sarcoma. Of the untreated series, only 2 developed tumors and 1 of these later retrogressed, while in the 8 surviving animals in the x-rayed lot, all developed large tumors which eventually caused death.

Result.—Normal series 22 per cent takes; x-rayed animals 100 per cent takes.

CONTROLS			IMMUNIZED			IMMUNIZED & X-RAYED		
1 st WEEK	2 nd WEEK	3 rd WEEK	1 st WEEK	2 nd WEEK	3 rd WEEK	1 st WEEK	2 nd WEEK	3 rd WEEK
+			+	-	-			
			-	-	-			
			+					+
+			+	+	-			
			-	-	-			
			+					
	+	+		D				
			+	-	-			
			+	-	-			

TEXT-FIG. 3. This chart shows the effect of x-ray on induced immunity. The silhouettes show the rate of growth of the inoculated cancer for 3 consecutive weeks after inoculation. The first group are the normal animals; the second, the immunized animals; and the third, animals that were immunized and given a series of small exposures to x-ray between the immunizing dose and the cancer inoculation.

Series B.—20 rats of the same lot were selected, and 10 of these were given the same amount of exposures to x-ray as in Series A. All were inoculated with equal sized grafts of the Jensen rat sarcoma. 8 of the normal animals survived, 3 of which number developed tumors, 1 retrogressing, however, after a period of growth. Among the x-rayed animals 6 lived and all of these developed large tumors, but 1 retrogressed after a period.

Result.—Normal animals 37 per cent takes; x-rayed animals 100 per cent takes.

Series C.—20 rats were selected and 10 of these were given x-ray exposures of 4 minutes' duration on 5 consecutive days. A white blood count and a differ-

ential were done before and 3 days after the x-ray treatment was discontinued. The circulating lymphocytes fell during this time on an average to 26 per cent of their former number, while the polymorphonuclear cells remained practically at the same level in actual numbers. All the 20 animals were inoculated with grafts of the Flexner-Jobling carcinoma of the rat. In the normal series only 2 animals developed tumors and 1 of these was of very slow growth. Among the x-rayed animals, all developed tumors which continued to grow till the death of the animals.

Result.—Normal animals 25 per cent takes; x-rayed animals 100 per cent takes.

Series D.—20 mice were used in this experiment, 10 being subjected to 1 minute exposures of x-ray on 10 consecutive days. All the animals were then inoculated with grafts of a transplantable mouse carcinoma. Among the normal animals, 4 out of the 9 surviving developed tumors which progressed till the death of the animal, while the other 5 recovered completely. In the x-rayed series 7 out of 9 surviving mice developed tumors.

Result.—Normal animals 44 per cent takes; x-rayed animals 77 per cent takes.

It will be noted that tumors have been used in this experiment at periods when they were giving a low percentage of takes, so as to make the test more rigid. We have also by this same method successfully transplanted spontaneous tumors to x-rayed animals when like grafts in normal animals failed to grow. They act, however, much as the heteroplastic tissues,¹⁰ retrogressing as the lymphoid tissue regenerates. With more carefully regulated dosage of x-ray it seems likely that uniform takes could be obtained in grafts from the spontaneous as well as from the transplantable tumors.

DISCUSSION.

The facts as they stand from these experiments are (1) that a marked lymphocytosis arises after inoculation with cancer in both the naturally immune animals and in the animals with induced immunity; and (2) when the lymphocytosis is prevented by a previous exposure of the animal to x-ray the resistant state is abolished. All the evidence points, therefore, towards the lymphocyte as a necessary factor in the immunity processes, and the finding offers a probable explanation of the results of Apolant who found that it was more difficult to render splenectomized animals immune to cancer than intact ones.¹¹

¹⁰ Murphy, Jas. B., *loc. cit.*

¹¹ Apolant, H., *Ztschr. f. Immunitätsforsch., Orig.*, 1913, xvii, 219.

Moreover, it has been noted that splenectomized animals exhibit less resistance to the growth of inoculated cancer than do intact ones giving both a higher percentage of takes and more rapidly growing tumors. This fact had been interpreted as the result of removal of one of the chief organs for antibody formation. But as no circulating antibodies have ever been demonstrated for cancer, it would seem more probable in the light of our work that the lymphocytic reaction has been interfered with by the removal of one of the principal lymphoid organs. We are not prepared, however, at the present time to discuss the mechanism of the lymphoid action in cancer immunity, or to do more than state that among the several possibilities that present themselves the data at hand are insufficient to establish one agency above all others.

SUMMARY.

The refractory state to transplanted cancer, induced by the subcutaneous injection of defibrinated blood, is accompanied in every case by a definite lymphoid crisis in the blood. The rise of lymphocytes is not present during the interval between the immunizing injection and the cancer inoculation but comes on sharply within twenty-four hours of the introduction of the cancer graft. In control animals where the graft leads to a definite take there is no such lymphoid response, but in instances of natural immunity the phenomenon is similar to that seen in artificially induced immunity, though the period of rise is often delayed for several days or a week.

The lymphoid crisis is not merely an accompanying factor in the immune period; it is essential to the process. This is demonstrated by the fact that destruction of the lymphocytes by x-ray is accompanied by the loss of natural or induced resistance to the growth of inoculated cancer.

The polymorphonuclear cells show a tendency to increase in the animals with growing tumors, but further study will be necessary before any conclusions regarding them can be drawn.

THE EFFECT OF PREVIOUS INTRAVENOUS INJECTION OF PNEUMOCOCCI UPON EXPERIMENTAL LOBAR PNEUMONIA PRODUCED BY THE METHOD OF INTRABRONCHIAL INSUFFLATION.

By B. S. KLINE AND S. J. MELTZER.

*(From the Department of Physiology and Pharmacology of The Rockefeller
Institute for Medical Research.)*

We wish to report a result obtained in a series of experiments in which the effect of intravenous injections of pneumococci upon subsequent experimental pneumonia was studied. Forty-nine dogs were used in these experiments. Observations were made upon the extent and morphology of the pneumonic process, the leukocytic reaction, the occurrence of agglutinins and the presence of living organisms in the blood and in the lungs. In one series, the animals were given intravenously each day for five days 0.7 c.c. of a broth culture of pneumococci per kilo of body weight. In another series graduated doses were given, beginning with 0.07 c.c. per kilo of body weight and gradually increased to the fifth and last injection of 0.7 c.c. per kilo. In both series on the sixth day, the dogs were given an intrabronchial injection of pneumococci. With groups of these animals, control dogs were also injected intrabronchially with pneumococci. The animals were killed at intervals from nineteen to forty-nine hours after the intrabronchial injection.

We wish to confine our present report to one significant result namely: the rapid disappearance of the organisms from the pneumonic lungs of the animals previously injected intravenously with pneumococci.

In previous experiments reported from this laboratory living organisms were observed in the lungs of dogs with experimental lobar pneumonia as late as the third day after the beginning of the

process. The results of the lung cultures in the present experiments may be seen from the charts.

It is evident from these charts that previous intravenous injections of pneumococci bring about a destructive effect upon the organisms in the pneumonic lung—the organisms disappear from these lungs much sooner than from the lungs of animals not previously treated.

CHART I.

Lung Cultures in Dogs Killed in Less than 36 Hours.

Controls.		Intravenously Injected.	
Hours.	Colonies.	Hours.	Colonies.
27	Innumerable	28 ½	0
30 ½	"	21 ½	0
29 ½	"	20	0
33	"	30	0
34	"	34 ½	0
31 ½	"	33 ½	1
20	+	30 ½	1
23	Very many	21 ½	1
19	Many	20 ½	3
23	12 + ¹	27	18
22 ½	0	29	75
		30	103
		24	Very many
		17	+
		20	Innumerable
		14 found dead	"

¹ Growth in water of condensation.

CHART II.

Lung Cultures in Dogs Killed in Less than 50 Hours.

Controls.		Intravenously Injected.	
Hours.	Colonies.	Hours.	Colonies.
42	Innumerable	45	0
41	Very many	46	0
49	Many	42	0
42	"	43	0
44 ½	"	43	0
48	+	41 ½	0
47	+	46 ½	0
46	25	46	0
45	3	47	0
48	0	49	0
		47 ½	13
		46	Very many

THE RELATION OF THE PURGATIVE ACTION OF MAGNESIUM SULPHATE TO PERISTALSIS, AND THE GENERAL LAW OF CROSSED INNERVATION.*

By S. J. MELTZER, M.D.

(From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research.)

While the subject I intend to discuss in particular is the inhibitory rôle of magnesium salts in its relation to the production of intestinal peristalsis, my chief object is to set forth certain general views I hold now, and have held for many years, on the subject of inhibition in general and the rôle which it plays in the mechanism of all sorts of movements in the animal organism. I shall begin the discussion of the relation of magnesium salts to peristalsis by calling attention to two sets of facts which seem to contradict each other.

It is a well-known and well-established fact that magnesium sulphate, or Epsom salts, is one of the most efficient purgatives. There have been many discussions as to the cause of the action of saline purgatives. According to one view, which has undergone many variations in its details, the cause of the purgation consists in the power of the saline to bring about an accumulation of liquid within the lumen of the intestine. We need not discuss here the nature and origin of this liquid. For our purpose it suffices to say that no matter from what cause liquid accumulates within the intestine, its mere presence would not cause purgation without the mediation of peristaltic movements of the intestinal canal. It is a known fact that the presence of large quantities of liquid within a paralyzed intestine does not lead to evacuation. According to another view which in recent years has been brought forward again by pupils of Jacques Loeb, the cause of action of certain purgative salts con-

* Read at the meeting of the Association of American Physicians, 1914.

sists directly in their ability to stimulate the neuro-muscular apparatus of the peristaltic mechanism. According to all views, then, peristaltic movements are indispensable for purgation, and therefore magnesium sulphate as an efficient purgative is expected to increase, or at least not to inhibit, the peristaltic movements of the intestines. I hardly need add that under the term peristalsis is generally meant a progressive movement of a vermiform character, which in our special case, means a vermiform movement of the gastro-intestinal canal running in an aboral direction.

Now in apparent opposition to the mentioned fact, I have to enumerate a number of observations which were safely established in our laboratory in the course of the last few years. In experiments carried out by Auer and myself several years ago, in which the behavior of the intestines of rabbits was observed in a warm bath of normal saline, we found that intravenous injections of magnesium salts stop peristaltic movements promptly. Usually not much peristaltic movement of the intestines can be seen, when the abdomen of a rabbit is opened. In order to observe the effect of magnesium sulphate, peristaltic movements had to be brought on by artificial means: by intravenous injections of sodium sulphate, barium chlorid, physostigmin or ergot, or by the destruction of the spinal cord. The peristaltic movements brought on by these means were sometimes of a violent character. All these peristaltic movements were either completely inhibited, or at least greatly reduced, by an intravenous injection of a solution of magnesium sulphate (or magnesium chlorid). In a later series of experiments, carried out by Don R. Joseph and myself, a method was employed in which the peristaltic movements could be studied in animals whose abdomens were closed. At one sitting the abdomen of rabbits was opened under proper anesthesia, and catheters, bearing at one end a small balloon, were inserted into the lower part of the duodenum and into the stomach; stomach, duodenum and abdomen were then properly closed. In the following days the protruding end of each catheter was connected with a graphic apparatus and tracings representing the peristaltic movements of the stomach and the duodenum were obtained. There were in nearly all cases good, spontaneous movements of the stomach and the duodenum. We have, then, estab-

lished beyond a doubt that the action of magnesium sulphate on the stomach and the duodenum is, under all circumstances, only of an inhibitory character. Whether the peristaltic movements were spontaneous in origin, or were brought about or intensified by barium chlorid or other substances, an intravenous injection of magnesium sulphate, even in an isotonic concentration, invariably caused an immediate inhibition, or at least an unmistakable reduction, of the peristalsis, lasting for some time after the injection has ceased.

These experimental results were obtained, as I stated before, by intravenous injections of the magnesium salts. I am now in a position to state, however, that the same inhibitory results can be readily obtained when the magnesium solutions are introduced into the lumen of the intestine. Auer and I found that magnesium salts, when introduced into the lumen of the intestine, are readily absorbed and may cause respiratory paralysis and death of the animals within a short time after the injection. Recently I have studied the effect of such a direct introduction of magnesium salts into the lumen of the intestine on the behavior of the peristaltic movements of the latter. I found that the intestine becomes practically paralyzed. I shall mention here one striking result. When the trachea of an animal is clamped and the animal thus killed by asphyxia, the exposed intestines show more or less strong peristaltic movements. When, however, a solution of magnesium salts is previously introduced into the lumen of the intestine, no part of the gastro-intestinal canal shows movements worth mentioning during or after the asphyxia.

These various experimental observations show unmistakably that magnesium salts, whether given intravenously or introduced into the lumen of the intestine, not only do not cause any peristalsis of the intestine, but positively inhibit it, when it is present. The puzzling question which now presents itself is this: If magnesium sulphate causes a suppression, an inhibition of the peristaltic movements, and only an inhibition, how can it bring on purgation in which, as we have seen above, peristaltic movement of the intestine is an indispensable factor?

To answer this question I shall have to enter into an analysis of inhibition and the rôle it plays in peristalsis. All know what is

meant by inhibition. The best known type of it is the phenomenon with which all are acquainted; it is the fact that the stimulation of the end of the vagus which goes to the heart stops the beating of that organ. The heart is a muscle. A nerve going to a muscle we usually term a motor nerve. Stimulation of such a nerve causes contraction of the muscle pertaining to it. But stimulation of the end of the vagus which goes to the heart stops the heart from beating. That is inhibition. Numerous other examples and other forms of inhibition are now well known. One of them is, that stimulation of the end of the splanchnic nerve which goes to the intestines, stops peristalsis of the intestine; it inhibits the peristalsis. Here we have some evidence that the phenomenon of inhibition may be connected with the peristalsis of the intestines. However, the most striking fact that inhibition is actually concerned in the peristaltic movements of the digestive tract was made by us thirty-four years ago in our studies of the function of deglutition. We then first established that after each beginning of a deglutition various parts of the esophagus develop a peristalsis at various intervals, according to their distances from the tongue and pharynx. For instance, the peristalsis of the cervical part of the esophagus sets in about two seconds after the beginning of deglutition; the esophagus in the upper part of the thorax begins to contract about three seconds, the part of the esophagus above the diaphragm begins to contract about six seconds, after the deglutition. These data were obtained from observations on my own esophagus. We discovered in the course of these studies that, when the deglutitions are repeated, say in less than every three seconds, the upper part of the thoracic esophagus does not contract after each deglutition, but only after the last deglutition has taken place. The same applies for the cervical as well as the lower part of the esophagus. In other words, with each deglutition which sends down an impulse for a contraction of the peristalsis, also an inhibitory impulse is sent down along the esophagus—for the purpose of inhibiting any possible contraction which may be present in any part of the esophagus, due to a previous act of deglutition. I may say here that each deglutition inhibits also the presence of a tonus in the esophagus. This physiological phenomenon reminds one of the precautions which are

taken when an express train is sent off; along the entire road telegraphic messages are sent, to keep the tracks free. That is the sense of the inhibitory wave which runs ahead of the peristaltic contraction to keep the track free.

Some thirty years ago I tried to generalize this point of view, to apply it to all functions of the body which require definite movements. I stated then, that, for instance, for the extension of the leg it is necessary that the flexors should be inhibited from being contracted simultaneously, or from remaining in a state of tonus. At that time I illustrated the actual occurrence of such antagonistic relations by the behavior of the function of respiration; during each inspiration the contraction of the expiratory muscles is inhibited, and during each expiration the diaphragm and other inspiratory muscles are suppressed, inhibited.

I wish to dwell a little longer on that subject. You probably know that these relations of flexors and extensors, as I anticipated them many years ago, have been brilliantly studied since 1895 by Sherrington, who terms the relations of flexors and extensors "reciprocal innervation." That designation is for these specific relations proper enough, because just as the flexors become inhibited, when the extensors have to contract, the extensors also become inhibited when the flexors are contracting. It is, then, a truly *reciprocal* innervation process. But when we apply these relations to a canal in which movements go on only in one direction, there is, of course, only one-half of this process. It is always only the lower part of the canal which has to be relaxed, when the upper part is contracting. A true antiperistalsis does not exist. In order to comprise all these phenomena under one head, I term the relations under discussion as "contrary innervation." It produces simultaneously a contraction in one part and an inhibition in another—in the contrary part. That means, two parts which are concerned in a single function are simultaneously innervated in a contrary way. The upper part of the canal is contracting in order to move the contents downward and the lower part is relaxing in order to remove any obstacle to this motion.

We shall now turn back to the digestive tract. I shall mention here first that, on the basis of these considerations, we discovered

long ago that with each act of deglutition the tonus of the cardia becomes inhibited—to open the gate into the stomach for the entrance of the contents which are swallowed down. In recent years Joseph and I found that during each contraction of the pyloric part of the stomach an inhibition of the movements of the duodenum takes place. Cannon and Lieb found that during a peristaltic contraction of the esophagus a simultaneous relaxation of the stomach makes its appearance. Finally, Lyman has observed that the colon becomes motionless and relaxed while the material is driven through the ileocolic sphincter by peristalsis in the ileum. All these observations have evidently that one background in common, namely, that for the purpose of moving downward the contents of the gastro-intestinal canal, the distal part of the various sections of the canal become inhibited, while the adjoining proximal (upper) part of the corresponding section gets into a state of contraction—a manifestation of the Law of Contrary Innervation.

Of greater interest to our problem is the instructive observation which was made by Bayliss and Starling some seventeen years ago, namely, that a stimulation of the intestines at any place causes a contraction above the point of stimulation and a relaxation or inhibition of the part of the intestine below the point of stimulation. Bayliss and Starling called this phenomenon the Law of the Intestines. But it is evident that this phenomenon is the law not only of the intestines, but of the entire digestive canal and that it is, furthermore, a part of the phenomenon which I term “Contrary Innervation.”

The outcome of the foregoing facts under consideration, bearing on the intestinal peristalsis, is evidently this: A peristaltic movement does not consist merely in a vermiform downward moving contraction; peristalsis is made up of two simultaneous phenomena: a contraction of the upper part and a relaxation or an inhibition of the lower part of the section of the gastro-intestinal canal, engaged in the act of moving downward some of its contents.

Bayliss and Starling have shown that this dual response of the intestine to a single stimulus takes place even when the intestines are freed from their extrinsic innervation. They thought that the process is of a myogenic origin; but Magnus has shown conclusively

that it is connected with Auerbach's plexus, the myenteric plexus, located between the longitudinal and circular sheaths of the fibers of the intestinal muscularis. We may, therefore, formulate our conclusion in the following terms: A stimulation of the myenteric plexus produces simultaneously a double effect, a contraction of the muscle fibers above, and an inhibition of any contractions below the point of stimulation.

I shall now turn my attention to the relation of magnesium sulphate to the phenomenon of inhibition. About sixteen years ago, when studying the effect of intracerebral injection of various salts, I came across the following surprising fact: While the injection into the cerebrum of many other salts produced characteristic convulsions, the injection of a few drops of a 5 per cent. solution of magnesium sulphate caused the rabbit to turn over on one side and remain lying in a state of stupor and relaxation. With a long standing interest in the phenomenon of inhibition it was quite natural for me to interpret the stuporous and relaxed condition of the animal as possibly being a state of inhibition. In the many studies of the action of salts on the animal organism to be found in the physiological and pharmacological literature, the effects observed and studied were always those of the exciting action of the salts. It was from the point of view that possibly the magnesium ion in the body is chiefly concerned in the inhibitory processes, that we started our various studies on the effects of magnesium. It became a working hypothesis; we had not yet come across some definite, weighty facts which are capable of disproving this hypothesis. As to the relations of magnesium salts to the gastro-intestinal canal, it has been stated before, that in the studies of Auer, Joseph and myself no other effect was found but that of inhibition of the peristaltic movements of the intestine.

We come now to our original question; it is this: On the one hand, it is certain that magnesium salts cause purgation, a process in which peristalsis must be a factor; on the other hand, the extensive experimental studies revealed the unmistakable fact that magnesium salts inhibit intestinal peristalsis. How can we reconcile these apparently contradictory facts? My answer to this question, given merely in a general way, is this: 1. The phenomenon of

peristalsis does not consist alone in a contraction of a certain part, but in a contraction of the proximal and *relaxation of the distal part* of a section of the intestine. 2. The effect of magnesium sulphate on peristalsis may consist chiefly in its relation to the inhibitory part of the phenomenon of peristalsis.

As I have just indicated, my answer to the central question shall be of a general character; I am unwilling for the present, to enter on definite statements as to how magnesium affects the peristalsis. I am confident that details which I may venture to state now will have to be modified by facts which future experimental investigations are sure to bring to light. I shall, however, try to continue the analysis by discussing briefly a few points which may have some bearing on the subject.

1. We have seen above that a stimulation of any part of the intestine causes by means of the intrinsic myenteric plexus (and probably also by the aid of the extrinsic innervation), a contraction above and a relaxation below. How strong the contraction of the part above may be and how intense is the inhibition of the part below, and how far it may extend, depends on a variety of circumstances. In the first place, we must here, as everywhere, distinguish between *irritability* and *stimulation*. Increase of irritability alone can bring out no action without the incident of a stimulus. The irritability of the concerned innervation may be low or high and may concern the exciting and inhibitory phases in various degrees. For instance, magnesium salts may increase exclusively or essentially the irritability of the inhibitory phase, while other salts, for instance, sodium sulphate, may increase preferably or exclusively, the irritability of the exciting phase. There is good reason for the assumption that the increase of irritability occurs through the circulation; but we have no definite evidence that this may not occur also by mere action on the periphery.

Again, stimulation may affect preferably the exciting phase or the inhibitory. But we are rather inclined generally to assume that a stimulus affects, as a rule, both the exciting and the inhibitory phases simultaneously. We are also inclined to assume that the point of attack of a stimulus is to be found at the periphery. But there is no evidence against the assumption that stimulation may take place

also directly through the circulation. We need hardly add that a stimulus may be weak or may be strong.

Taking all these points together, it can be seen at a glance how variable the effects of some substances on the intestine may be. With a low irritability of certain sections of the intestine, and with a comparatively low stimulus from the periphery, the effect may be altogether circumscribed, and the result may be, not peristalsis, but only "segmentation," the effect of each stimulus not being an intense contraction above and not an extensive relaxation below. With an increased irritability and with a moderately strong stimulus, there may be a fair contraction above and a fairly extensive relaxation below, and the result will be a peristalsis extending over a fairly large section. With a strongly increased irritability of both phases and with a strong stimulus, the result may be a rapid peristalsis, running through the entire intestine within a very short time, a condition which we termed "peristaltic rush."

2. A brief discussion of peristaltic rush will not be amiss here. As was just stated, by "peristaltic rush" we designate the phenomenon of a rapid peristaltic movement running downward through the entire small intestine within a fraction of a minute. This phenomenon was observed for the first time by Van Bram-Houckgeest some forty years ago in rabbits dying from asphyxia. He termed it *Rollbewegungen*. Several years ago Auer and I succeeded in producing this phenomenon practically at will in living rabbits. The method we used consisted in injecting intravenously antagonistic substances; that is, one substance which favors contractions of the gut, and another which favors the inhibition of such contractions; for instance, ergot and magnesium sulphate.

3. Our experimental studies of the inhibitory effect of magnesium salts on peristalsis were made either by administering magnesium sulphate intravenously or by injecting it into the lumen of the small intestine. However, when administering magnesium sulphate by mouth (as for the purpose of purgation), while the salt passes through the stomach it meets with sodium chlorid and some carbonates. It is therefore possible, and even probable, that parts of the magnesium sulphate become converted into sodium sulphate and magnesium carbonate. Sodium sulphate is soluble and is readily

absorbed. From the experiments of MacCallum and of Auer we know that sodium sulphate when given intravenously is apt to bring on some peristalsis. Magnesium carbonate is not soluble and remains within the intestine probably entirely unabsorbed. We know that magnesium carbonate is capable of acting as a purgative. The insoluble carbonate acts possibly within the intestine in two ways: on the one hand, it attracts liquid into the lumen of the intestine thus distending it, and, on the other hand, the carbonate is likely to serve as a chemical stimulus directly on the mucosa. At any rate, it is not impossible that that part of the magnesium sulphate which has been converted into a carbonate acts within the intestine as a stimulus calling forth peristaltic movements. On the other hand, the part of the magnesium sulphate which is converted into sodium sulphate may be the means of increasing the irritability of the exciting phase of the peristalsis; it may serve also as a stimulus to the neuromuscular apparatus. The remaining unconverted portion of the magnesium sulphate increases the irritability of the inhibitory phase of peristalsis. Now we have seen above that by the administration of antagonistic substances, that is, substances, one of which increases the exciting phase, while the other increases the inhibitory phase of peristalsis, are capable of calling forth a "peristaltic rush," that is, a peristaltic wave which runs rapidly through the entire small intestine within a short time. We can therefore easily conceive that the purgative action of magnesium sulphate, when administered by the mouth, results from the fact that a part of it acts by increasing the inhibitory phase of peristalsis, while other parts, by being converted partly in sodium sulphate and partly into magnesium carbonate, increase the irritability of the exciting phase of peristalsis and serve as an inciting stimulus to start peristaltic movements. The result is the development, after some time, of partial and perhaps complete peristaltic rushes.

I wish to repeat again that I offer this detailed analysis of the action of magnesium sulphate merely as a preliminary hypothesis. Future experimental work will throw more light on these questions.

4. In a few words, my general conception of the various effects which may result from the simultaneous actions of exciting and inhibitory factors on the functions of the animal body is as fol-

lows: The resultant effects of the antagonistic actions can be of a threefold nature which may be designated as *tonus*, *rhythm*, and *peristalsis*.

1. The resultant effect of the antagonistic actions may be *manifest at the same place and at the same time*. This is a *tonus*. The degree and the character of the tonus depend on the preponderance of one or the other factor.

2. The effect of both antagonistic factors may be *manifest at the same place, but at different, alternating times*. It is then a *rhythm*; contraction alternates with inhibition.

3. Both effects of the antagonistic factors may be present *at the same time, but manifest themselves simultaneously in different parts*. The resultant is then, as far as the intestines and other muscular tubes are concerned, a *peristalsis*; a contraction above and an inhibition below.

RETENTION IN THE CIRCULATION OF DEXTROSE IN
NORMAL AND DEPANCREATIZED ANIMALS, AND
THE EFFECT OF AN INTRAVENOUS INJECTION
OF AN EMULSION OF PANCREAS
UPON THIS RETENTION.*

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The content of dextrose in the circulating blood of normal animals is almost constant, it amounts to about 0.1 per cent. The carbohydrates of the foodstuffs form the main source of dextrose in the body. On their way from the digestive tract the carbohydrates are transformed into various forms of saccharides; but all are finally converted largely into glycogen, which is stored up mostly in the liver. The blood obtains its supply of dextrose from the glycogen of the liver, and distributes it among the tissues of the body according to their demand for it. In the normal animal none of the dextrose escapes through the kidneys. Accordingly the constancy of the amount of dextrose in the blood is regulated by a mechanism which controls either of the two factors; the *supply* of or the *demand* for it.

In diabetes the dextrose content of the blood is higher than normal, is variable in amount, and, when it is sufficiently high, dextrose escapes through the kidneys. The cause of the increase of the blood dextrose, or hyperglycaemia, may be found either in a decrease in the demand of the tissues for dextrose, that is, the tissues burn dextrose less readily than in normal conditions; or in an increase in the supply, that is, the liver supplies the blood with more dextrose than in normal conditions. Both possibilities have groups

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of followers who have brought forward numerous experiments in support of either of the respective theories.

It was experimentally established years ago that the complete removal of the pancreas brings on a severe hyperglycaemia, leading to considerable glycosuria and to the death of the animal. Here we are confronted again with the question, whether the presence of the pancreas in the normal animal favors the burning of the dextrose by the tissues, or whether it restricts the supply of dextrose to the blood by the liver. But there also arose another question, namely, whether the effect of the removal of that gland was due to a removal of a controlling substance, or, whether it was due, in consequence of the operation, to an interference with the nervous mechanism which controls the supply of the dextrose. In case of the first alternative, namely, that the resulting hyperglycaemia is due to a removal of some substance, normally secreted by the pancreas, we have to assume that that substance belongs to that group of substances which are provided by glandular and other tissues for the proper maintenance of functions of the animal body, i.e., *internal secretions*, since a complete suppression of the external secretions of the pancreas never leads to hyperglycaemia or diabetes.

We wish to report briefly results of several series of experiments which are likely to shed some light upon these questions. Most of our experimental series have one feature in common, namely, that the blood was provided with dextrose directly and not by way of the liver. In one series of experiments large quantities of dextrose (20 cc. of a 20% solution of dextrose per kilo body weight) were injected intravenously into normal dogs. It was found by us, as it was found by some investigators before us, that the injected large surplus of dextrose disappeared quite rapidly from the circulation. *Ninety minutes after the infusion, the dextrose content of the blood reached nearly the same level which it had before the injection.* About one-half of the injected quantity of dextrose left the circulation through the kidneys; the other half went into the tissues. In nephrectomized animals also the dextrose returned to the previous level in the same length of time. *Here we have a striking example of the ability and readiness of the circulation of a normal animal to rid itself of a large surplus of dextrose.*

In another series of experiments similar injections of dextrose were made into animals, the pancreas of which was removed by an operation a day or two before the dextrose infusion. The sample of blood taken from the animal before the intravenous injection of dextrose was strongly hyperglycaemic. It was then found that in these experiments *the dextrose content of the sample of blood, which was taken ninety minutes after the end of the dextrose injection, was at least twice as high as that of the sample taken before the injection.* Apparently the removal of the pancreas interfered with the power of the circulation to rid itself readily of a surplus of dextrose. The possible ability of the liver to provide the circulation with a greater supply of dextrose in the absence of the pancreas can certainly be no important factor in these results. Our experiments show, then, *that in the presence of the pancreas the circulation rids itself easily of intravenously introduced dextrose; but that it is unable to do it satisfactorily in the absence of the pancreas.* On the basis of these facts is it not plausible to assume further that the pancreas exerts exactly the same influence, when the dextrose in the circulation is being supplied in some other manner than by a burette into a vein, for instance, by the liver through its connections with the circulation? Or, in other words, do not our experiments make the assumption plausible that pancreatic hyperglycaemia is due to the fact that *in the absence of the pancreas the circulation is unable to dispose properly of the dextrose which it receives in normal amounts from the liver?*

In a third series, the experiments were again made by injecting dextrose intravenously in depancreatized dogs. But in these cases a strained pancreatic emulsion was added to the dextrose solution, and in some instances the infusion of the pancreatic emulsion was continued for some time after the infusion of the dextrose was finished. We present here merely a preliminary communication; we have not yet mastered all the details of this part of the investigation. But there seems to be no doubt about the nature and validity of the main result, which is this: In such experiments, *ninety minutes after the end of the dextrose infusion, the dextrose content of the blood is again at about the same level as it was before the infusion of the dextrose.* Apparently the presence of a pancreatic emulsion within

the blood helps the circulation to get rid of the surplus of dextrose injected intravenously. In these experiments it is evident that the *effect of the pancreas can be only of a chemical and not of a nervous nature*, or, in other words, it is the internal secretion of the pancreas which helps the circulation to get rid of the surplus of dextrose.

To the above results we wish now to add the following preliminary statement derived from three experiments. *In depancreatized animals the blood of which has shown a marked hyperglycaemia, an intravenous infusion of a pancreas emulsion brought the dextrose content of the blood to 0.09% and even 0.08%.* The hyperglycaemia returned next day and could be reduced again to the normal level by an infusion of pancreas emulsion.

CONCLUSIONS.

In normal animals the circulation possesses the ability to get rid readily of a surplus of dextrose injected intravenously. In the absence of the pancreas this ability of the circulation is impaired. This ability can be temporarily restored by an intravenous injection of a pancreas emulsion.

Furthermore, an intravenous injection of a pancreas emulsion is capable of reducing the hyperglycaemia due only to depancreatization to a normal level of the dextrose content of the blood.

As to the nature of the factors which may constitute the ability or inability of the circulation to get rid of a certain degree of surplus of the dextrose content of the blood, we are not willing to discuss it at this stage of our investigation. We are rather bent upon seeing how many more facts we shall be enabled to bring to light on the basis of the hypothesis which set us to work on these problems.

THE QUATERNARY SALTS OF HEXAMETHYLENE- TETRAMINE.

III. MONOHALOGENACYLATED AROMATIC AMINES AND THEIR HEXAMETHYLENETETRAMINIUM SALTS.¹

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In the second of this series of papers we have described a number of compounds obtained from the halogenacetyl derivatives of various benzylamines. The present paper will deal with those obtained from the halogenacetyl derivatives of aromatic amines in which the amino group is situated directly on the nucleus. The scope of the work and the great variety of amino compounds employed demonstrate how practically limitless in this direction is the development of hexamethylenetetraminium salts.

As described in greater detail in the first paper, the addition of the halogen compound to hexamethylenetetramine was accomplished by the use of molecular equivalents of the constituents in a small volume of dry, boiling chloroform. Unless otherwise stated the products separated during the reaction and were filtered off and washed well with dry chloroform and dry acetone. Purity of the final product is assured by the use of pure materials. Otherwise it is, as a rule, difficult or impossible to recrystallize the salts without danger of decomposition. These substances are usually readily soluble in water, in which, particularly on boiling, they

¹ For parts I and II of this series see this *Journal*, xx, pp. 659 and 685, 1915.

decompose with separation of precipitates. These, on purification, proved to be the methyleneglycyl derivatives of the amines employed. A description of these substances and a discussion of their significance is reserved for a later communication.

In the course of the work occasion arose for the preparation of new intermediate products which will be described at length in the experimental part.

EXPERIMENTAL.

The method most frequently used for the chloroacetylation was as follows: One molecular equivalent of the base was dissolved in toluene or benzene. To the solution was added an excess of two-normal sodium hydroxide solution, and then, drop by drop, with cooling and vigorous shaking, a solution of one and one-quarter molecules of chloroacetyl chloride in about two volumes of dry benzene. On short standing, the chloroacetyl derivative usually crystallized out, the precipitation being facilitated in a number of cases by the addition of ligroin. In dealing with the more soluble and more easily accessible amines, two molecules of base to one of chloride were used, thus dispensing with the need of the aqueous alkali solution. Important deviations from the above method are indicated as they occur.

Chloroacetylaniline and Hexamethylenetetramine.—Equimolecular quantities of the components were boiled in dry chloroform solution for one-half hour. The salt separated on chilling and scratching, and was obtained in practically quantitative yield on completing the precipitation with an equal volume of dry acetone. It forms glistening micro-crystals, readily soluble in the cold in water and absolute alcohol. It darkens at 155° and melts with decomposition at $158-9^{\circ}$.

0.2987 gm. of substance required 9.6 cc. AgNO_3 Solution I.²

Calculated for $\text{C}_{14}\text{H}_{20}\text{ON}_4\text{Cl}$: Cl = 11.45 per cent.

Found: Cl = 11.31 per cent.

Bromoacetylaniline and Hexamethylenetetramine.—The solution of the components in boiling chloroform soon set to a solid cake.

² 1 cc. = 0.00352 gm. Cl; 0.00793 gm. Br; 0.01259 gm. I.

Heated in a capillary tube, the salt first darkens, then melts at 163° with decomposition. It is less soluble than the chloride.

0.2043 gm. of substance required 5.8 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{14}\text{H}_{20}\text{ON}_4\text{Br}$: Br = 22.55 per cent.

Found: Br = 22.52 per cent.

Chloroacetylmethylaniline was found to have the melting point given by Grothe³ (70°), and not that recorded by Kuhara and Chikashigé⁴ (61°) or Bischoff⁵ (48°).

Chloroacetylmethylaniline and Hexamethylenetetramine.—The chloroform solution was evaporated to small bulk after two hours' boiling, and the salt precipitated by adding dry acetone. Three extractions of one hour each with boiling, dry acetone were found necessary to remove the chloroform of crystallization from the product. It melts at $168-73^{\circ}$ with gas evolution, and is easily soluble in chloroform and alcohol. It is extremely soluble in water.

0.1984 gm. of substance required 6.3 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{14}\text{H}_{20}\text{ON}_4\text{Cl}$: Cl = 10.96 per cent.

Found: Cl = 11.18 per cent.

Chloroacetyldiphenylamine⁶ and Hexamethylenetetramine.—The salt, as prepared in chloroform solution, forms hair-like needles, decomposing at $158-61^{\circ}$, and retaining a molecule of chloroform of crystallization even at 100° *in vacuo*.

0.2037 gm. of substance required 4.01 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{20}\text{H}_{24}\text{ON}_4\text{Cl} \cdot \text{CHCl}_3$: Cl = 7.02 per cent.

Found: Cl = 6.93 per cent.

An attempt to remove the chloroform by boiling the salt with dry acetone led to the substitution of two molecules of acetone for the molecule of chloroform, the resulting product decomposing at $151-62^{\circ}$.

0.2075 gm. of substance required 4.12 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{20}\text{H}_{24}\text{ON}_4\text{Cl} \cdot 2\text{CH}_3\text{COCH}_3$: Cl = 7.07 per cent.

Found: Cl = 6.99 per cent.

³ W. Grothe: *Chem. Zentralbl.*, lxxi, pt. ii, p. 1268, 1900.

⁴ M. Kuhara and M. Chikashigé: *Am. Chem. Jour.*, xxvii, p. 1, 1902.

⁵ C. A. Bischoff: *Ber. d. deutsch. chem. Gesellsch.*, xxxiv, p. 2125, 1901.

⁶ H. Frerichs: *Chem. Zentralbl.*, lxxiv, pt. ii, pp. 103-4, 1903.

This conclusion was confirmed by the absence of the odor of chloroform on dissolving the substance in water, and by the fact that the distillate from the aqueous solution yielded a copious precipitate of iodoform, negative results being obtained on controls run with chloroform and the hexamethylenetetraminium salt of chloroacetylaniline.

Chloroacetylphenylglycinanilide.—The benzene layer from the chloroacetylation of the base was evaporated to a syrup *in vacuo*, water added, and the remaining benzene removed by further distillation. The water was poured off from the tarry residue, which was taken up in alcohol and made to crystallize by the cautious addition of water. Yield: poor. The substance was recrystallized twice from 95 per cent alcohol, then twice from benzene, from which it separates with benzene of crystallization. This is given off very slowly *in vacuo* at room temperature. After finally heating at 100° , the compound melts at $118-20^{\circ}$ (corrected) with preliminary softening. It is readily soluble in chloroform, difficultly in ether.

0.1461 gm. of substance (Kjeldahl) required 9.45 cc. $\frac{N}{8}$ HCl.

0.2010 gm. of substance⁷ required 12.50 cc. AgNO₃ Solution II.⁸

Calculated for C₁₆H₁₈O₂N₂Cl: N=9.26 per cent; Cl=11.72 per cent.

Found: N=9.06 per cent; Cl=11.57 per cent.

Chloroacetylphenylglycinanilide and Hexamethylenetetramine.—The presence of a little chloroform in the salt, even after drying to constant weight *in vacuo* at the temperature of boiling alcohol over sulphuric acid, was recognized by its swimming about on solution in water, and by the odor evolved in so doing. The salt melts at $157-8^{\circ}$.

0.1939 gm. of substance required 7.83 cc. AgNO₃ Solution II.

Calculated for C₂₂H₂₇O₂N₄Cl: Cl=8.10 per cent.

Found: Cl=7.51 per cent.

Chloroacetyl- ω -anilinoacetophenone.—6.8 grams of anilinoacetophenone (phenacylaniline) were suspended in about 25 cc. of benzene and 3.8 grams of chloroacetyl chloride added. After the first evolution of heat, the mixture was warmed on the water bath

⁷ Hydrolysis with alcoholic sodium hydroxide.

⁸ 1 cc. = 0.00186 gm. Cl; 0.004192 gm. Br.

for two hours, hydrochloric acid being evolved. On cooling a small residue was filtered off, and the filtrate treated with ligroin, precipitating an oil which rapidly solidified. This was recrystallized from dilute alcohol, forming prisms with a slight greenish yellow tinge and melting at $116-7.5^{\circ}$. Yield: 7.3 grams. Recrystallized again from methyl alcohol, the substance forms practically colorless, hexagonal plates, melting at $117-8^{\circ}$ (corrected) with slight softening at 116.5° . It is difficultly soluble in cold methyl alcohol and dry ether.

0.3579 gm. of substance (Kjeldahl) required 12.45 cc. $\frac{N}{N}$ HCl.

Calculated for $C_{16}H_{14}O_2NCl$: N = 4.87 per cent.

Found: N = 4.87 per cent.

Chloroacetyl- ω -anilinoacetophenone and Hexamethylenetetramine.

—The salt is somewhat soluble in chloroform, easily in water and absolute alcohol, and melts with decomposition at $169-9.5^{\circ}$.

0.2246 gm. of substance required 5.41 cc. $AgNO_3$ Solution I.

Calculated for $C_{22}H_{20}O_2N_6Cl$: Cl = 8.29 per cent.

Found: Cl = 8.48 per cent.

Chloroacetyl-o-toluidine and Hexamethylenetetramine.—The salt melts with decomposition at $148-9^{\circ}$ and is easily soluble in water.

0.3189 gm. of substance required 9.85 cc. $AgNO_3$ Solution I.

Calculated for $C_{10}H_{12}ON_6Cl$: Cl = 10.96 per cent.

Found: Cl = 10.87 per cent.

Bromoacetyl- ω -o-toluidinoacetophenone.—14.6 grams of phenacyl-o-toluidine were dissolved in about 100 cc. of benzene and 15 grams of bromoacetyl bromide added. The reaction occurred at once with separation of the hydrobromide of the base. On boiling for three-quarters of an hour, hydrobromic acid was evolved and a clear solution obtained. About two-thirds of the benzene were then removed and the bromoacetyl derivative was precipitated by the addition of ligroin to the warm solution. After recrystallization from 85 per cent alcohol it formed thick, diamond-shaped plates melting at $107-7.5^{\circ}$. Yield: 17.3 grams. The compound is extremely soluble in chloroform, less so in benzene, and difficultly in ether.

0.3361 gm. of substance (Kjeldahl) required 9.9 cc. $\frac{N}{8}$ HCl.

Calculated for $C_{11}H_{14}O_2NBr$: N = 4.05 per cent.

Found: N = 4.13 per cent.

Bromoacetyl- ω -o-toluidinoacetophenone and Hexamethylenetetramine.—On heating a chloroform solution of the components to boiling, the reaction proceeded spontaneously with formation of a clear solution. After one-half hour's heating, a small amount of what was probably hexamethylenetetraminium bromide was filtered off and an equal volume of dry acetone added. The salt was then precipitated as a caseous mass by means of dry ether, becoming crystalline on rubbing. It was purified by warming with dry acetone, in which it proved to be insoluble when once crystalline. The salt melts with decomposition at $175-6^\circ$ and is difficultly soluble in water and chloroform.

0.2017 gm. of substance required 4.24 cc. $AgNO_3$ Solution I.

Calculated for $C_{22}H_{24}O_2N_6Br$: Br = 16.43 per cent.

Found: Br = 16.68 per cent.

Chloroacetyl-*m*-toluidine was prepared from *m*-toluidine in toluene, normal potassium hydroxide solution, and chloroacetyl chloride. The compound separated in good yield on adding ligroin and cooling. Whereas Kuhara and Chikashigé⁴ give 141° as the melting point, our preparation, after recrystallization first from dilute alcohol then toluene, melted at $90-1.5^\circ$ (corrected).

0.2550 gm. of substance (Kjeldahl) required 13.80 cc. $\frac{N}{8}$ HCl.

Calculated for $C_9H_{10}ONCl$: N = 7.63 per cent.

Found: N = 7.58 per cent.

Chloroacetyl-m-toluidine and Hexamethylenetetramine.—After short boiling, the chloroform solution of the components suddenly set to a solid cake. This was disintegrated and boiled for ten minutes longer with a little more dry chloroform. The salt darkens above 170° and melts at $173-4^\circ$. It is very soluble in water.

0.1622 gm. of substance required 9.10 cc. $AgNO_3$ Solution II.

Calculated for $C_{10}H_{12}ON_6Cl$: Cl = 10.96 per cent.

Found: Cl = 10.43 per cent.

Chloroacetyl-p-toluidine and Hexamethylenetetramine.—As obtained from chloroform solution, the salt was not entirely soluble in

water. It was purified by boiling with dry acetone, whereupon it dissolved completely, although rather difficultly, in water. It melts at $176-8^{\circ}$ with decomposition.

0.2744 gm. of substance required 8.36 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{10}\text{H}_{22}\text{ON}_4\text{Cl}$: $\text{Cl} = 10.96$ per cent.

Found: $\text{Cl} = 10.72$ per cent.

Chloroacetyl-m-4-xylylidine⁴ and Hexamethylenetetramine.—The salt forms silky needles, melting at $171-2^{\circ}$.

0.2170 gm. of substance required 6.3 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{10}\text{H}_{20}\text{ON}_4\text{Cl}$: $\text{Cl} = 10.52$ per cent.

Found: $\text{Cl} = 10.22$ per cent.

Chloroacetyl- ψ -cumidine⁴ and Hexamethylenetetramine.—The salt separated from chloroform in fine needles, and was purified by boiling with hot ethyl acetate. It melts with decomposition at $170-1^{\circ}$ and is easily soluble in water and alcohol.

0.2183 gm. of substance required 6.14 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{11}\text{H}_{20}\text{ON}_4\text{Cl}$: $\text{Cl} = 10.08$ per cent.

Found: $\text{Cl} = 9.90$ per cent.

Chloroacetyl- α -naphthylamine and Hexamethylenetetramine.—On adding several volumes of dry acetone to the chloroform reaction mixture, the salt separated after a few moments in rosettes of needles, melting at $164-6^{\circ}$ with decomposition and difficultly soluble in water.

0.2849 gm. of substance required 7.97 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{18}\text{H}_{20}\text{ON}_4\text{Cl}$: $\text{Cl} = 9.86$ per cent.

Found: $\text{Cl} = 9.85$ per cent.

Chloroacetyl- β -naphthylamine⁹ and Hexamethylenetetramine.—The salt melts at $177-8^{\circ}$ with decomposition and is difficultly soluble in water.

0.2612 gm. of substance required 7.15 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{18}\text{H}_{20}\text{ON}_4\text{Cl}$: $\text{Cl} = 9.86$ per cent.

Found: $\text{Cl} = 9.64$ per cent.

Chloroacetyl-*o*-chloroaniline was prepared by Schwalbe, Schulz,

⁹ T. B. Johnson and W. K. Walbridge: *Jour. Am. Chem. Soc.*, xxv, p. 483, 1903.

and Jochheim¹⁰ by two methods differing from the one outlined above. After chloroacetylation, the toluene layer was washed successively with dilute hydrochloric acid, water, dilute sodium hydroxide, and water, and evaporated to small bulk. The substance separated as glistening needles after the addition of ligroin. Recrystallized from benzene, it softens at 71.5° and melts at 73.5–4.° (corrected). The above mentioned authors give 67° as the melting point.

Chloroacetyl-o-chloroaniline and Hexamethylenetetramine.—The salt separates from the boiling chloroform solution in the form of glistening hairs. It melts with decomposition at 158–60° and is somewhat difficultly soluble in water.

0.1722 gm. of substance required 9.37 cc. AgNO₃ Solution II.

Calculated for C₁₄H₁₀ON₂Cl₂: Cl = 10.30 per cent.

Found: Cl = 10.12 per cent.

p-Bromochloroacetylaniline. This was prepared by Frerichs⁶ from *p*-bromoaniline. We have found that the substance is easily obtained by treating a solution of chloroacetylaniline in acetic acid drop by drop with one molecular equivalent of bromine dissolved in acetic acid. The bromo derivative separates out in part on standing, and is more completely precipitated on dilution of the solvent with water. After recrystallization from acetic acid or dilute alcohol, it conforms to the description given by Frerichs.

p-Bromochloroacetylaniline and Hexamethylenetetramine.—In order to obtain a product entirely soluble in water, it was necessary to boil out the crude salt with a fresh portion of chloroform. It forms prismatic needles containing chloroform of crystallization, a part of which is retained even on drying to constant weight *in vacuo* at the temperature of boiling alcohol over sulphuric acid, or on boiling out with dry acetone.

0.1783 gm. of substance (Kjeldahl) required 20.54 cc. $\frac{N}{10}$ HCl.

0.2476 gm. of substance required 5.59 cc. AgNO₃ Solution I.

Calculated for C₁₄H₁₀ON₂ClBr· $\frac{3}{2}$ CHCl₃: N = 16.05 per cent; Cl = 8.13%.

Found: N = 16.13 per cent; Cl = 7.95%.

¹⁰ C. G. Schwalbe, W. Schulz, and H. Jochheim: *Ber. d. deutsch. chem. Gesellsch.*, xli, p. 3791, 1908.

The original crystals apparently contained $\frac{1}{3}$ CHCl_3 , as a portion, dried between filter papers, lost 9.47 per cent on drying to constant weight as above. Calculated for $\frac{1}{3}$ CHCl_3 : Loss = 9.87 per cent.

2, 4, 6-Tribromochloroacetylaniline.—9 grams of tribromoaniline and 2.7 cc. of chloroacetyl chloride were heated in about 100 cc. of toluene in an oil bath at $110-20^\circ$ for about four hours. On cooling, the deposit of needles was filtered off and recrystallized from xylene. Yield: 8.5 grams. The substance melts at $221-2^\circ$ (corrected) with partial decomposition, and is difficultly soluble, in the cold, in the usual solvents.

0.3350 gm. of substance (Kjeldahl) required 8.1 cc. $\frac{N}{10}$ HCl .

Calculated for $\text{C}_6\text{H}_3\text{ONClBr}_3$: $\text{N} = 3.45$ per cent.

Found: $\text{N} = 3.39$ per cent.

An attempt to prepare the hexamethylenetetraminium salt resulted only in the isolation of a product consisting largely of hexamethylenetetraminium chloride.

m-Iodochloroacetylaniline.—The substance separated from the toluene solution when prepared as described in the introduction. It forms slender, felted needles, which, when recrystallized first from dilute alcohol, then toluene, melt at $121.5-2.5^\circ$ (corrected). It is fairly readily soluble in cold absolute alcohol, benzene, and chloroform.

0.3534 gm. of substance (Kjeldahl) required 12.65 cc. $\frac{N}{10}$ HCl .

Calculated for $\text{C}_6\text{H}_4\text{ONClI}$: $\text{N} = 4.74$ per cent.

Found: $\text{N} = 4.77$ per cent.

m-Iodochloroacetylaniline and Hexamethylenetetramine.—The salt turns greenish above 170° and melts with decomposition at 175° . It is difficultly soluble in water.

0.1922 gm. of substance required 8.08 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{11}\text{H}_{11}\text{ON}_4\text{ClI}$: $\text{Cl} = 8.14$ per cent.

Found: $\text{Cl} = 7.82$ per cent.

5-Iodochloroacetyl-o-toluidine.—This was prepared from 5-iodo-2-toluidine¹¹ similarly to the above aniline derivative. Recrystallized first from dilute alcohol, then from chloroform, it forms glis-

¹¹ H. L. Wheeler and L. M. Liddle: *Am. Chem. Jour.*, xlii, p. 501, 1909.

tening needles melting at $161.5-2.5^{\circ}$ (corrected) with slight preliminary softening. It is more soluble in the cold in ethyl acetate, absolute alcohol, and acetone than in the other usual organic solvents.

0.3185 gm. of substance (Kjeldahl) required 10.20 cc. $\frac{N}{10}$ HCl.

Calculated for C_8H_9ONCl : N = 4.53 per cent.

Found: N = 4.48 per cent.

5-Iodochloroacetyl-o-toluidine and Hexamethylenetetramine.—

Combination of the components in boiling chloroform proceeded slowly. The salt forms minute, flat prisms which melt with decomposition at $190-2^{\circ}$ and are rather difficultly soluble in water.

0.2045 gm. of substance required 8.37 cc. $AgNO_3$ Solution II.

Calculated for $C_{10}H_{11}ON_4Cl$: Cl = 7.89 per cent.

Found: Cl = 7.61 per cent.

*m-Nitrochloroacetylaniline*¹² and Hexamethylenetetramine.—The pale yellow salt melts at $162-3^{\circ}$.

0.2264 gm. of substance required 6.3 cc. $AgNO_3$ Solution I.

Calculated for $C_{10}H_9O_2N_4Cl$: Cl = 10.00 per cent.

Found: Cl = 9.8 per cent.

p-Nitrochloroacetylaniline.—24 grams of commercial *p*-nitraniline were dissolved in commercial acetone and filtered from impurities. 7.4 cc. of chloroacetyl chloride were then slowly added, with stirring and cooling. Without removing the precipitated hydrochloride, dilute hydrochloric acid was added in excess, and then, to the resulting clear solution, hot water until crystallization started. On cooling rapidly, the chloroacetyl derivative separated in glistening crystals. Yield: about 90 per cent of the theory. Recrystallized from acetic acid, it forms pale brownish yellow prisms which melt at $182-4^{\circ}$ with preliminary softening. Deutsch¹³ describes the substance as sintering above 124° and melting at 152° .

0.4770 gm. of substance (Kjeldahl) required 43.95 cc. $\frac{N}{10}$ HCl.

Calculated for $C_8H_7O_2N_4Cl$: N = 13.06 per cent.

Found: N = 12.90 per cent.

¹² T. B. Johnson and W. B. Cramer: *Jour. Am. Chem. Soc.*, xxv, p. 490, 1903.

¹³ E. Deutsch: *Jour. f. prakt. Chem.*, lxxvi, p. 360, 1907.

m-Nitrochloroacetyl-*p*-toluidine and Hexamethylenetetramine.—

The pale yellow salt melts with decomposition at $163-4^{\circ}$ and turns bright yellow when added to water, dissolving slowly and giving a yellow solution. The salt is also somewhat soluble in alcohol.

0.2368 gm. of substance required 6.49 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{10}\text{H}_{11}\text{O}_3\text{N}_3\text{Cl}$: Cl = 9.59 per cent.

Found: Cl = 9.65 per cent.

m-Chloroacetylaminodimethylaniline.—Groll's¹⁴ method for the preparation of *m*-nitrodimethylaniline proved to be very unsatisfactory, the *p*-isomer being obtained as the chief product.

m-Aminodimethylaniline (Schuchardt) was chloroacetylated in benzene solution. In order to complete the separation of the chloroacetyl derivative, about one-half volume of ligroin was added to the benzene layer and the mixture allowed to stand over night in the ice box. Yield: 50–80 per cent of the theory. After two recrystallizations from 95 per cent alcohol, the compound forms grayish rhombs, softening at 101° and melting at $101.5-2.5^{\circ}$ (corrected). It couples readily with diazo compounds, and is very soluble in the cold in acetone, less so in acetic acid, and difficultly in alcohol, benzene, and ether.

0.1614 gm. of substance (Kjeldahl) required 15.2 cc. $\frac{N}{10}$ HCl.

Calculated for $\text{C}_{10}\text{H}_{13}\text{ON}_2\text{Cl}$: N = 13.18 per cent.

Found: N = 13.19 per cent.

m-Chloroacetylaminodimethylaniline and Hexamethylenetetramine.—The green, viscous solution obtained by boiling the components in dry chloroform was diluted with several volumes of dry acetone, precipitating the salt as a pale bluish, micro-crystalline powder. Boiling for one hour in dry acetone removed most of the color. The purified salt melts with decomposition at $151-2^{\circ}$ and is readily soluble in water, but difficultly in chloroform.

0.2220 gm. of substance required 6.59 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{10}\text{H}_{12}\text{ON}_3\text{Cl}$: Cl = 10.05 per cent.

Found: Cl = 10.45 per cent.

p-Aminodimethylaniline.—A portion of the *p*-aminodimethyl-

¹⁴ A. Groll: *Ber. d. deutsch. chem. Gesellsch.*, xix, p. 198, 1886.

aniline used in this and other experiments was furnished us through the courtesy of the Farbwerke Höchst. We desire to take this opportunity also to thank this firm for their kindness in supplying us with this and other intermediate products of the dyestuff industry. In preparing additional quantities of *p*-aminodimethylaniline, the following method was found to be very rapid and serviceable: 100 grams of *p*-nitrosodimethylaniline were added in small amounts to a warm solution of 450 grams of stannous chloride in 900 cc. of concentrated hydrochloric acid, occasional cooling being necessary. The mixture was finally warmed for one-half hour on the water bath, cooled, and saturated with hydrochloric acid gas at 0° in order to complete the precipitation of the double tin salt. This was filtered off, dissolved in water, the solution covered with a layer of ether, and, with cooling, made very strongly alkaline with 50 per cent sodium hydroxide solution. The upper layer was removed and the alkaline solution shaken out several times with ether. After removal of the ether, the residue was fractionated *in vacuo*. Yield: 72 grams, boiling at 146–8° at 24 mm., and melting at 38–41°.

p-Chloroacetylaminodimethylaniline.—After chloroacetylation of the base in benzene solution, using chloroacetyl chloride and aqueous sodium hydroxide, the separation of the chloroacetyl derivative was completed by the addition of ligroin. Recrystallized from benzene, with bone-blackening, the compound answered the description given by Rupe and Všeťčka.¹⁵ Yield: 74 per cent of the theory.

p-Chloroacetylaminodimethylaniline and Hexamethylenetetramine.—The salt forms glistening plates containing chloroform of crystallization and melting at 145–8°. After drying to constant weight *in vacuo* at the temperature of boiling alcohol, the substance melts at 171–2° with decomposition and preliminary darkening, and still retains chloroform. It is easily soluble in water, the solution smelling of chloroform and giving an orange-red color with ferric chloride. During the analysis by the Volhard method, a deep pink color developed, but it was found possible to destroy this by adding a large excess of concentrated nitric acid, thus permitting the determination of the end-point.

¹⁵ H. Rupe and J. Všeťčka: *Ann. d. Chem.*, ccc, p. 75, 1898.

0.2100 gm. of substance required 5.23 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{16}\text{H}_{28}\text{ON}_4\text{Cl} \cdot \frac{1}{2}\text{CHCl}_3$: $\text{Cl}^- = 8.60$ per cent.

Found: $\text{Cl}^- = 8.77$ per cent.

p-Aminodiethylaniline was prepared from *p*-nitrosodiethylaniline hydrochloride in the same way as the dimethyl compound, except that the reduction was started in the cold and the double tin salt was filtered off after chilling, saturation of the reaction mixture with hydrochloric acid not being resorted to. The yield was the same as in the case of the dimethylamino compound. The substance is a pale yellow oil which boils at $139.5\text{--}40.5^\circ$ at 9.5–10 mm. and melts below room temperature.

p-Chloroacetylaminodiethylaniline.—This was prepared in the same way as the dimethylamino compound, and with approximately the same yield. Recrystallized from dilute alcohol, it forms faintly pink, delicate, lustrous needles, melting at $83\text{--}4^\circ$ (corrected), and easily soluble in ether, chloroform, benzene, and acetone.

0.1993 gm. of substance (Kjeldahl) required 16.0 cc. $\frac{N}{10}$ HCl.

Calculated for $\text{C}_{12}\text{H}_{17}\text{ON}_2\text{Cl}$: $\text{N} = 11.65$ per cent.

Found: $\text{N} = 11.48$ per cent.

p-Chloroacetylaminodiethylaniline and Hexamethylenetetramine.—The mechanical stimulus of filtration was sufficient to induce crystallization of the salt from the reaction mixture. The precipitation was made more complete by the addition of two volumes of dry acetone. The compound forms glistening micro-crystals melting with decomposition at $159\text{--}60^\circ$ and dissolving readily in water and alcohol. The same precautions must be observed in the analysis as in the case of the dimethylamino analog.

0.2120 gm. of substance required 5.65 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{16}\text{H}_{28}\text{ON}_4\text{Cl}$: $\text{Cl} = 9.32$ per cent.

Found: $\text{Cl} = 9.38$ per cent.

p-Nitrosodipropylaniline Hydrochloride.—28 grams of dipropylaniline were dissolved in a mixture of 60 grams of concentrated hydrochloric acid and 125 grams of ice and water. A concentrated aqueous solution of 12.2 grams of sodium nitrite was then added, drop by drop, with stirring and chilling. After stirring for about half an hour, the nitroso hydrochloride was salted out and filtered

off. A small portion was dissolved in hot absolute alcohol, bone-blackened, filtered, and precipitated by dry ether as a greenish yellow micro-crystalline powder. The salt dissolves readily in water, and when heated rapidly, darkens and decomposes violently at about $160-5^{\circ}$. It is a powerful irritant to the mucous membrane.

0.4317 gm. of substance gave 0.2560 gm. AgCl.

Calculated for $C_{12}H_{18}ON_2 \cdot HCl$: Cl = 14.61 per cent.

Found: Cl = 14.67 per cent.

p-Aminodipropylaniline.—The crude, red-brown nitroso hydrochloride was reduced as in the case of the dimethylaniline analog. In this way, from 28 grams of dipropylaniline were obtained 15.5 grams of *p*-amino compound as a somewhat viscous, pale yellow oil of which most boiled at $155.5-6.5^{\circ}$ at 6 mm. It does not solidify in a freezing mixture, is miscible in all proportions with absolute alcohol, and is slightly soluble in hot water.

0.1546 gm. of substance (Kjeldahl) required 15.85 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{12}H_{18}N_2$: N = 14.58 per cent.

Found: N = 14.37 per cent.

p-Chloroacetylaminodipropylaniline.—The compound separated from the benzene layer of the reaction mixture after adding ligroin and letting stand over night in the ice box. Recrystallized from 95 per cent alcohol, it forms practically colorless prisms softening at 120° and melting at $121-1.5^{\circ}$ (corrected). Yield: 14 grams from 15 grams of base.

0.2691 gm. of substance (Kjeldahl) required 20.13 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{14}H_{21}ON_2Cl$: N = 10.43 per cent.

Found: N = 10.48 per cent.

p-Chloroacetylaminodipropylaniline and Hexamethylenetetramine.—On one occasion the salt separated incompletely from the chloroform solution as an oil, in another preparation as a pasty mass. In both cases, about three volumes of dry acetone were added in order to complete the precipitation and induce crystallization. The compound forms minute platelets containing solvent of crystallization which is driven off *in vacuo* at the temperature of boiling alcohol. The salt then melts at $165-8^{\circ}$ to a tar, and is difficultly soluble in water, easily in chloroform, absolute alcohol, and dilute acids.

0.2185 gm. of substance required 10.26 cc. AgNO_3 Solution II.¹⁶

Calculated for $\text{C}_{20}\text{H}_{28}\text{ON}_4\text{Cl}$: Cl = 8.68 per cent.

Found: Cl = 8.73 per cent.

p-Chloroacetylaminooethylbenzylaniline. — *p*-Aminoethylbenzylaniline was prepared according to the method of Schultz, Rohde, and Bosch.¹⁷ The chloroacetyl derivative separates from the benzene layer of the reaction mixture as an oil which rapidly solidifies. After letting stand over night in the ice box, the substance was filtered off and recrystallized from 95 per cent alcohol, with bone-blackening. It forms pale brownish micro-prisms melting at $106-8^\circ$ to a brown liquid and is readily soluble in benzene. Yield: poor.

0.2128 gm. of substance (Kjeldahl) required 14.01 cc. $\frac{N}{10}$ HCl.

Calculated for $\text{C}_{17}\text{H}_{19}\text{ON}_2\text{Cl}$: N = 9.26 per cent.

Found: N = 9.23 per cent.

p-Chloroacetylaminooethylbenzylaniline and Hexamethylenetetramine.—The salt separated incompletely from the chloroform solution as an oil, which was converted into crystalline form by the addition of dry acetone to the mixture. It forms glistening micro-platelets melting with decomposition at $163-4^\circ$ and is very difficultly soluble in water. An aqueous solution may be obtained, however, by adding a molecular equivalent of dilute hydrochloric acid.

0.2060 gm. of substance required 4.97 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{22}\text{H}_{21}\text{ON}_4\text{Cl}$: Cl = 8.21 per cent.

Found: Cl = 8.49 per cent.

p-Chloroacetylaminooazobenzene.—(See formula I.) The substance was filtered from the toluene layer of the reaction mixture and recrystallized from 95 per cent alcohol, with bone-blackening. Yield: about 50 per cent. Recrystallized from toluene, it forms sheaves of golden-yellow needles which soften at 155° and melt at 159.5° (corrected). The compound is soluble in hot chloroform and dissolves in sulphuric acid with a brown-orange color.

0.1409 gm. of substance gave 19.45 cc. N (752 mm. and 23.2°).

Calculated for $\text{C}_{14}\text{H}_{12}\text{ON}_4\text{Cl}$: N = 15.36 per cent.

Found: N = 15.30 per cent.

¹⁶ The pink color produced by the indicator may be temporarily destroyed by the addition of concentrated nitric acid.

¹⁷ G. Schultz, G. Rohde, and E. Bosch: *Ann. d. Chem.*, cccxxxiv, p. 262, 1904.

p-Chloroacetylaminoozobenzene and Hexamethylenetetramine.—The mechanical stimulus of filtering the deep red chloroform solution was sufficient to induce crystallization. The orange-yellow salt melts with decomposition at $153-4^{\circ}$. It is difficultly soluble in water, the solution dyeing silk a light yellow shade. The stability of the aqueous solution and the solubility are increased by the addition of a small amount of aqueous sodium carbonate. It turns red under sulphuric acid and dissolves with an orange color.

0.2756 gm. of substance required 6.63 cc. AgNO_3 Solution I.¹⁸

Calculated for $\text{C}_{10}\text{H}_9\text{ON}_2\text{Cl}$: Cl = 8.57 per cent.

Found: Cl = 8.47 per cent.

Chloroacetylaminoozotoluene (*o*-Tolueneazochloroacetyl-*o*-toluidine).—This was prepared from aminoazotoluene (from *o*-toluidine) in the same way and with the same yield as chloroacetylaminoozobenzene. Recrystallized first from toluene with the aid of a little ligroin, and then from absolute alcohol, it forms pale salmon-colored needles which soften at 168.5° and melt at $171-2.5^{\circ}$ (corrected). The compound is difficultly soluble in the cold in the usual organic solvents, easily on warming, and gives a brown-red solution in sulphuric acid.

0.1417 gm. of substance (Kjeldahl) required 14.32 cc. $\frac{N}{10}$ HCl .

Calculated for $\text{C}_{10}\text{H}_{10}\text{ON}_2\text{Cl}$: N = 13.93 per cent.

Found: N = 13.89 per cent.

Chloroacetylaminoozotoluene and Hexamethylenetetramine.—The orange-yellow salt melts at $167-9^{\circ}$ to a red-brown liquid. It is somewhat soluble in chloroform and absolute alcohol and dissolves much more readily in water than the preceding unsubstituted homolog. It gives an orange color with sulphuric acid. The aqueous solution dyes silk a pale yellow shade.

0.2637 gm. of substance required 6.07 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{10}\text{H}_{10}\text{ON}_2\text{Cl}$: Cl = 8.03 per cent.

Found: Cl = 8.10 per cent.

β -Naphthaleneazochloroacetyl- β -naphthylamine. — The product obtained from 14.8 grams of β -naphthaleneazo- β -naphthylamine

¹⁸ Before titrating the substances of this type, dilute nitric acid was added to the hot solution. The mixture was cooled and the precipitate filtered off.

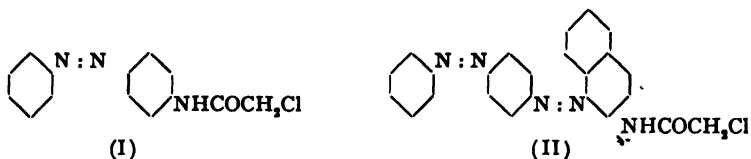
(aminoazo- β -naphthalene) as in the above cases was filtered off, washed with toluene, and recrystallized from acetone. Yield: 10.9 grams. Recrystallized first from toluene with the aid of a little ligroin and then from amyl alcohol, it melts with decomposition at $181.5-2.5^\circ$ (corrected). The compound forms bright orange, hair-like needles, easily soluble in chloroform, difficultly in alcohol, and soluble in sulphuric acid with a deep violet color.

0.1387 gm. of substance gave 14.1 cc. N (757 mm. and 25.3°).

Calculated for $C_{22}H_{16}ON_4Cl$: N = 11.25 per cent.

Found: N = 11.24 per cent.

The hexamethylenetetramine addition product of the chloroacetyl derivative proved to be insoluble in water and could not be purified. Before this result was obtained we had already under way the preparation of other substances of this type, but owing to the unpromising properties of the above hexamethylenetetramine derivative this line of work was dropped. For purposes of record, however, we give below a description of the substances which had been prepared.



Benzeneazobenzeneazochloroacetyl- β -naphthylamine. — Benzeneazobenzeneazo- β -naphthylamine (benzene-*p*-disazobenzene- β -naphthylamine) was chloroacetylated as in the previous cases. After cooling, the product (see formula II) was filtered off, washed with toluene, and recrystallized from this, with bone-blackening. Yield: about 60 per cent of the theory. The compound forms brick-red micro-needles which darken above 188° and melt at 190° (corrected) with decomposition. It is very difficultly soluble in absolute alcohol, more readily in chloroform, and gives a red-brown solution with sulphuric acid.

0.1221 gm. of substance gave 18.4 cc. N (746 mm. and 22.4°).

Calculated for $C_{24}H_{18}ON_4Cl$: N = 16.38 per cent.

Found: N = 16.63 per cent.

o-Tolueneazo-o-tolueneazo-β-naphthylamine.—17.2 grams of aminoazotoluene (from *o*-toluidine) were stirred into 25 cc. of concentrated hydrochloric acid, after which the mixture was diluted with water, cooled with ice, and slowly diazotized with a little less than the theoretical amount of sodium nitrite. The solution of the diazonium salt was filtered into a well stirred suspension of β-naphthylamine hydrochloride obtained by rapidly chilling a solution of 10.9 grams of the amine in hot, dilute hydrochloric acid containing 9 cc. of concentrated acid. The mixture was turbined for one-half hour, filtered, and the solid washed successively with dilute hydrochloric acid, water, dilute aqueous ammonia, and water. The crude product was dissolved in hot acetone, bone-blackened, and crystallized by adding hot water and cooling. Yield: 13.8 grams. Recrystallized from toluene, the compound forms glistening, dark violet-brown leaflets which melt at 174–7° and dissolve in toluene and acetone with a deep red color, and in sulphuric acid with a deep blue-green color. In concentrated hydrochloric acid it dissolves with difficulty with a deep blue color.

0.1295 gm. of substance gave 21.25 cc. N (754 mm. and 23.8°).

Calculated for $C_{24}H_{21}N_5$: N = 18.45 per cent.

Found: N = 18.18 per cent.

o-Tolueneazo-o-tolueneazochloroacetyl-β-naphthylamine. — The base was dissolved in warm toluene and chloroacetylated in the usual way after adding an excess of normal aqueous sodium hydroxide. Recrystallized twice from acetic acid and then from amyl alcohol, it melts to a tar at 122–6° with preliminary softening. It forms a dark brown micro-crystalline powder, gives a strong halogen test with copper wire, and yields deep red-brown solutions with chloroform, acetone, and benzene. The color in sulphuric acid is dark brown.

0.1220 gm. of substance gave 16.2 cc. N (755 mm. and 22°).

Calculated for $C_{24}H_{22}ON_2Cl$: N = 15.37 per cent.

Found: N = 14.87 per cent.

o-Tolueneazo-α-naphthylamine.—Although this substance has been used by previous workers¹⁹ and is easily prepared by diazotiz-

¹⁹ Compare R. Nietzki and R. Zehntner: *Ber. d. deutsch. chem. Gesellsch.*, xxvi, p. 145, 1893.

ing *o*-toluidine and coupling with α -naphthylamine in the usual manner, we were unable to find any description of it in the literature.²⁰ Recrystallized first from dilute alcohol and then ligroin, it forms rosettes of orange-red hairs melting at $97-7.5^\circ$ (corrected) with slight preliminary softening. It is very soluble in the usual organic solvents, and gives deep red solutions in sulphuric and acetic acids. The hydrochloride is very difficultly soluble in water.

0.1243 gm. of substance gave 17.45 cc. N (758 mm. and 22°).

Calculated for $C_{17}H_{18}N_2$: N = 16.09 per cent.

Found: N = 15.78 per cent.

o-Tolueneazo- α -naphthaleneazo- β -naphthylamine.—The above dye was diazotized, coupled, and basified as in the case of the corresponding aminoazotoluene derivative (see above). The yield was excellent. Recrystallized twice from amyl alcohol, the substance sinters above 140° and melts at about $173-8^\circ$. It forms a black-brown, micro-crystalline powder which is soluble in chloroform, acetone, and benzene with a purplish red color, and difficultly soluble in ether and absolute alcohol. It gives an intense blue color with sulphuric acid, dissolves with difficulty in dilute hydrochloric acid with a faint purple color, and is insoluble in dilute nitric and sulphuric acids. The fact that the compound burned with difficulty probably explains the low result on analysis.

0.1360 gm. of substance gave 19.45 cc. N (763 mm. and 21.3°).

Calculated for $C_{27}H_{24}N_4$: N = 16.87 per cent.

Found: N = 16.23 per cent.

As there seemed to be little hope for the preparation of water-soluble hexamethylenetetramine dyes from intermediates containing benzene and naphthalene radicals with neutral substituents only, it was decided to experiment with dyes containing salt-forming groups. The series of dyes described below was developed with this in view.

p-Acetaminobenzeneazodimethylaniline.—This compound was prepared by Meldola²¹ by the reduction and acetylation of the cor-

²⁰ After this paper had been sent to press we found a description of this substance by L. Casale and M. Casale-Sacchi (*Gazz. chim. ital.*, xlv, pt. ii, p. 395, 1914), who give the melting point as 95° .

²¹ R. Meldola: *Jour. Chem. Soc.*, xlv, p. 108, 1884.

responding nitro compound. As this method did not give good results in our hands, the following procedure was used: the diazonium solution from 19.8 grams of *p*-aminoacetanilide was slowly run into an alcoholic solution of 32.3 grams of dimethylaniline, with cooling and vigorous stirring. The separation of the orange-brown dye was completed by the addition of sodium acetate, after which the product was filtered off and washed with 50 per cent alcohol. Yield: 31.4 grams, melting at 223–7°. Recrystallized from 90 per cent alcohol, it melts at 226–7° and not at 217°, as stated by Meldola.

p-Aminobenzeneazodimethylaniline.—The above acetyl derivative was boiled for thirty-five minutes with about eleven parts of sulphuric acid (1:4), the mixture gradually assuming a thinner consistency as the hydrolysis proceeded. The solution was cooled and, with continued cooling, made slightly alkaline with aqueous ammonia. The red-brown, bulky precipitate was filtered off, washed with water and 50 per cent alcohol, and dried. Yield: 22.5 grams.

p-Chloroacetylaminobenzeneazodimethylaniline.—7.5 grams of amino compound were dissolved as well as possible in about 100 cc. of warm benzene and chloroacetylated in the usual manner with chloroacetyl chloride and normal sodium hydroxide solution. The dark brown precipitate was filtered off, washed well with toluene, water, and 50 per cent alcohol, and dried. Yield: 8.5 grams. Recrystallized from boiling isoamyl alcohol, in which the substance is difficultly soluble, it forms glistening, chocolate-brown microplates, soluble in chloroform and acetone. The solution in dilute mineral acids dyes silk a brownish yellow shade. When rapidly heated, the compound sinters at about 190° and melts at about 205°.

0.2097 gm. of substance (Kjeldahl) required 26.45 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{10}H_{11}ON_2Cl$: N = 17.71 per cent.

Found: N = 17.68 per cent.

p-Chloroacetylaminobenzeneazodimethylaniline and Hexamethylenetetramine.—The brown salt was washed well with chloroform and boiled with dry acetone. It melts at 167–8.5° and is difficultly soluble in water, more easily on adding a dilute mineral acid, the color of the solution changing from orange to deep purple. It dyes silk a brown-orange color.

0.1189 gm. of substance gave 25.4 cc. N (762 mm. and 20.5°).²²

0.3075 gm. of substance gave 0.1001 gm. AgCl.

Calculated for $C_{12}H_{22}ON_4Cl$: N = 24.53 per cent; Cl = 7.76 per cent.

Found: N = 24.33 per cent; Cl = 8.05 per cent.

p-Acetaminobenzeneazodiethylaniline.—This substance was prepared in the same way as the dimethyl homolog. Yield of crude product: 35 grams. 5 grams of this, recrystallized from 85 per cent alcohol, yielded 4.8 grams of glistening, orange-brown plates which form an orange-red powder when pulverized and soften at 186° to a semicrystalline mass which becomes entirely fluid at 192°. The solution in dilute hydrochloric acid dyes silk a brownish yellow shade.

0.1536 gm. of substance (Kjeldahl) required 19.77 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{18}H_{22}ON_4$: N = 18.06 per cent.

Found: N = 18.05 per cent.

p-Aminobenzeneazodiethylaniline.—30 grams of acetaminobenzene derivative were saponified as in the case of the dimethyl compound. The partly resinous product solidified on standing, and was ground up in a mortar and dried. Yield: 23.2 grams. Recrystallized first from benzene with the aid of an equal volume of ligroin, and then twice from 85 per cent alcohol, it forms dark brown crystals with a blue-black luster. These melt at 149–50° (corrected) with preliminary softening.

0.1529 gm. of substance (Kjeldahl) required 22.63 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{16}H_{20}N_4$: N = 20.89 per cent.

Found: N = 20.74 per cent.

p-Chloroacetylaminobenzeneazodiethylaniline.—15 grams of amino compound were partially dissolved in warm toluene and chloroacetylated as in the case of the dimethyl homolog. Precipitation of the chloroacetyl derivative was assisted by adding an equal volume of ligroin to the toluene solution before cooling. The product was combined with a small amount obtained by extracting the toluene-ligroin solution with 5 per cent sulphuric acid and making alkaline, and then recrystallized by bone-blackening in benzene and adding li-

²² Quantitative results were obtained only when the substance was mixed with lead chromate.

groin to the hot, filtered solution. Yield: 9.6 grams of chocolate-brown crystals, which sinter above 155° and melt at $160.5-2^{\circ}$. The solution in dilute mineral acids dyes silk a brown-orange shade.

0.1490 gm. of substance (Kjeldahl) required 17.27 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{18}H_{21}ON_4Cl$: N = 16.26 per cent.

Found: N = 16.25 per cent.

p-Chloroacetylaminobenzeneazodiethylaniline and Hexamethylenetetramine.—Precipitation of the brown-orange salt from the chloroform solution was completed by adding dry acetone and cooling in the ice box. The product melts at $172-3^{\circ}$ with preliminary sintering, is somewhat hygroscopic, and is easily soluble in water, yielding a brown-orange solution which dyes silk the same color.

0.2787 gm. of substance gave 0.0868 gm. AgCl.

Calculated for $C_{24}H_{30}ON_4Cl$: Cl = 7.32 per cent.

Found: Cl = 7.71 per cent.

p-Acetaminobenzeneazodipropylaniline.—15 grams of *p*-aminoacetanilide were diazotized and the solution was added, with stirring, to a cold solution of 17.7 grams of dipropylaniline in about 300 cc. of 85 per cent alcohol containing 20 grams of sodium acetate. During the coupling process, which took place rapidly, a little 95 per cent alcohol was added in order to prevent the separation of unchanged dipropylaniline. After fifteen minutes' stirring, the mixture was allowed to stand for one hour, whereupon the precipitation of the dye was completed by the addition of water. Yield: 26.4 grams. Recrystallized first from 95 per cent alcohol and then from toluene, the compound forms small, orange needles which, when rapidly heated, melt at 178° (corrected). In dilute hydrochloric acid it dyes silk a brownish yellow shade.

0.1227 gm. of substance gave 18.25 cc. N (760 mm. and 25.1°).

Calculated for $C_{20}H_{25}ON_4$: N = 16.56 per cent.

Found: N = 16.50 per cent.

p-Chloroacetylaminobenzeneazodipropylaniline.—The acetamino compound (23 grams) was hydrolyzed as in the previous examples, but as difficulty was experienced in obtaining the amino derivative in a crystalline state, the crude, resinous product was dissolved in ben-

zene and chloroacetylated in the usual manner with 100 cc. of two-normal sodium hydroxide solution and 5 cc. of chloroacetyl chloride. The benzene layer was evaporated to small bulk in the cold by means of a current of air, yielding, in all, 9.4 grams of red-brown micro-platelets. Recrystallized again in the same manner, the substance forms orange-brown aggregates of micro-crystals melting at $112-4^{\circ}$ (corrected) with preliminary softening and darkening. It is readily soluble in benzene and chloroform, less so in absolute alcohol.

0.1581 gm. of substance (Kjeldahl) required 16.67 cc. $\frac{N}{8}$ HCl.

Calculated for $C_{20}H_{20}ON_4Cl$: N = 15.03 per cent.

Found: N = 14.78 per cent.

p-Chloroacetylaminobenzeneazodipropylaniline and Hexamethylenetetramine.—The solid cake formed on boiling the components for forty minutes in dry chloroform solution was disintegrated, treated with about two volumes of dry acetone, and filtered off. The salt was then ground up in a mortar with dry acetone and washed well with this, being obtained finally in the form of orange-yellow micro-platelets which soften and sinter above 100° and melt completely at $180-1^{\circ}$. It is somewhat less soluble in water than the diethyl homolog and dyes silk a brown-orange shade.

0.3125 gm. of substance required 6.47 cc. $AgNO_3$ Solution I.²³

Calculated for $C_{20}H_{27}ON_4Cl$: Cl = 6.92 per cent.

Found: Cl = 7.29 per cent.

p-Acetaminobenzeneazoethylbenzylaniline.—The diazonium solution from 20 grams of *p*-aminoacetanilide was slowly run into a well stirred solution of 28 grams of ethylbenzylaniline in about 500 cc. of absolute alcohol containing 15 grams of sodium bicarbonate in suspension. As coupling took place very slowly, about 5 cc. of concentrated aqueous ammonia were added and the stirring continued about three hours. Aqueous ammonia was then added in slight excess, followed by about two volumes of water. This precipitated a dark colored oil which solidified almost completely on standing several days. The product was filtered off and dried *in vacuo* over sul-

²³ After dissolving the substance in fairly strong nitric acid and adding an excess of the silver nitrate solution, the mixture was heated at 100° until most of the dye was destroyed. The precipitate was filtered off and the silver determined gravimetrically in the filtrate.

phuric acid, after which it was extracted with cold ether and washed well with this. The substance was thus obtained in a yield of 23.7 grams as a chocolate-brown powder. Recrystallized from 85 per cent alcohol, it forms minute brown rhombs with a violet reflex. On drying *in vacuo* at the temperature of boiling alcohol, water is given off and the color changes to red-brown.

0.8248 gm. of substance lost 0.0230 gm.

Calculated for $C_{22}H_{24}ON_4 \cdot \frac{1}{2}H_2O$: $H_2O = 2.36$ per cent.

Found: $H_2O = 2.79$ per cent.

0.1819 gm. of dried substance (Kjeldahl) required 19.37 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{22}H_{24}ON_4$: $N = 15.05$ per cent.

Found: $N = 14.93$ per cent.

Recrystallized from benzene, the compound forms yellow-brown spears with a violet luster which melt at $148-50^\circ$ (corrected) with slight preliminary reddening and softening. These form an ochre powder when pulverized, and, in very dilute hydrochloric acid solution, dye silk a lemon-yellow shade.

The *amino derivative*, obtained by hydrolysis of the acetyl compound by boiling for one-half hour with ten parts of sulphuric acid (1:4), is best isolated by allowing the cooled solution to run slowly into well chilled and rapidly stirred dilute ammonia. In this way the base separates in the form of brown, amorphous flocks which coalesce to a thick resin on filtering. The crude product was taken up in benzene and used for the preparation of the chloroacetyl derivative.

p-Chloroacetylaminobenzeneazoethylbenzylamine.—150 cc. of normal sodium hydroxide solution were added to the above benzene solution and the mixture was thereupon treated with 6 cc. of chloroacetyl chloride, added in small portions, with vigorous shaking. The benzene layer was evaporated to small bulk *in vacuo* in the cold, depositing brown crystalline crusts which were recrystallized from toluene. Yield: 6.5 grams. The compound melts at $140-1.5^\circ$ (corrected) and is easily soluble in acetone and chloroform, difficultly in cold alcohol and toluene. It dissolves in acetic acid with a deep red color. The solution in dilute hydrochloric acid dyes silk a brownish yellow shade.

0.1367 gm. of substance gave 16.7 cc. N (764 mm. and 23.7°).

Calculated for $C_{22}H_{28}ON_4Cl$: N = 13.78 per cent.

Found: N = 13.73 per cent.

p-Chloroacetylaminobenzeneazoethylbenzylaniline and Hexamethylenetetramine.—After about twenty minutes' boiling the chloroform solution set to a solid cake. This was broken up, boiled ten minutes longer with a little more dry chloroform, filtered, and washed cautiously with dry chloroform in order to remove the brown color. Excessive amounts of solvent must be avoided, as the salt is somewhat soluble in chloroform. After grinding up with dry acetone, the residue forms ochreous micro-plates which gradually sinter above 110° and melt to a tar at 140°. The salt dissolves in water, forming an orange solution which dyes silk an orange-red color. It is also soluble in alcohol.

0.2588 gm. of substance required 4.53 cc. $AgNO_3$ Solution I.²⁴

Calculated for $C_{22}H_{28}ON_4Cl$: Cl = 6.48 per cent.

Found: Cl = 6.16 per cent.

All the above dialkylamino dyes dissolve in sulphuric acid with brownish orange colors and in dilute mineral acids with the formation of purple solutions.

m-Bromodiethylaniline.—Twenty grams of *m*-bromoaniline, 56 grams of ethyl iodide, 12 grams of sodium hydroxide, and about 50 cc. of absolute alcohol were heated at 100° in a pressure bottle for eight hours, with occasional shaking. Water was added, the precipitated oil taken up in ether, and the bases were extracted with 10 per cent sulphuric acid. They were then precipitated with sodium hydroxide, taken up with ether, dried, and the solution evaporated to small bulk. The residue was then warmed for three hours on the water bath with acetic anhydride in order to acetylate the primary and secondary amines. Ether was then added and the tertiary amine extracted by shaking with 10 per cent sulphuric acid. The base was precipitated with sodium hydroxide, taken up in ether, and the solution dried over potassium hydroxide and evaporated to small bulk. On fractionating *in vacuo* the substance was obtained in a

²⁴ The solution was warmed with nitric acid until pale yellow, after which an excess of the silver nitrate solution was added, the tar and silver chloride filtered off, and the titration completed as usual.

yield of 10.1 grams as a highly refracting, colorless liquid boiling at 139.5–142° (corrected) at 9–10 mm. It is heavier than water, has a slight aromatic odor, and solidifies in a freezing mixture, melting again at a low temperature.

0.1373 gm. of substance (Kjeldahl) required 6.22 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{10}H_{14}NBr$: N = 6.14 per cent.

Found: N = 6.35 per cent.

p-Acetaminobenzeneazo-2'-bromo-4'-diethylaminobenzene. — The dye was prepared similarly to the ethylbenzylaniline analog. A small amount of tar was obtained, which solidified on long keeping. Recrystallized twice from absolute alcohol, it forms minute, glistening rhombs which sinter and redden above 160° and melt at 163.5–4° (corrected). It readily forms highly supersaturated solutions and is very soluble in chloroform, less so in benzene, acetone, hot alcohol, and hot amyl alcohol. The dark violet solution in dilute hydrochloric acid dyes silk a deep yellow shade. The substance gives a strong Beilstein test and dissolves in sulphuric acid with a red-brown color.

0.1510 gm. of substance gave 19.3 cc. N (771 mm. and 22°).

Calculated for $C_{18}H_{21}ON_4Br$: N = 14.39 per cent.

Found: N = 14.62 per cent.

Benzeneazo-2'-chloroacetylamino-4'-dimethylaminobenzene. — This substance of the chrysoidine type was prepared by diazotizing and coupling 2.1 grams of aniline with a solution of 5 grams of *m*-chloroacetylaminodimethylaniline (page 140) in one molecular equivalent of 10 per cent hydrochloric acid, following the directions for the preparation of chrysoidine as given in Möhlau and Bucherer's *Farbenchemisches Praktikum*, p. 132 (Leipsic, 1908), except that the heating was omitted. Recrystallization of the crude product from absolute alcohol yielded 2.9 grams of scarlet tablets with a faint violet luster. The compound melts at 109.5–110° (corrected) with slight preliminary softening, is readily soluble in chloroform and benzene, and dissolves in sulphuric acid with a bright orange color. It is difficultly soluble in cold, dilute hydrochloric acid, the solution dyeing silk an orange shade.

0.1430 gm. of substance (Kjeldahl) required 17.9 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{14}H_{17}ON_4Cl$: N = 17.71 per cent.

Found: N = 17.53 per cent.

The hexamethylenetetraminium salt was obtained in poor yield and in a very impure condition.

4-Nitrobenzeneazo-2'-chloroacetyl-amino-4'-dimethylaminobenzene.

—The diazonium solution obtained from 3.25 grams of *p*-nitraniline was diluted to about 600 cc. To the well stirred solution were added, in small portions, 5 grams of finely powdered *m*-chloroacetylaminodimethylaniline, the solution becoming a dark violet-brown. After one-half hour's stirring, normal sodium acetate solution was added, drop by drop, until the reaction became neutral to Congo Red paper. After standing over night in the ice box, the violet-brown precipitate was filtered off, dried, and recrystallized from hot chloroform, in which it is very difficultly soluble. The substance was obtained in this way in the form of dark violet-brown, voluminous, triboelectric masses of micro-crystals. When rapidly heated it melts at 220° . It is difficultly soluble in the usual solvents, excepting hot nitrobenzene, from which it separates in dark crystals with a metallic green reflex. It gives a bright red color with sulphuric acid and dissolves with difficulty in dilute hydrochloric acid with a brownish violet color.

0.1642 gm. of substance (Kjeldahl) required 22.45 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{18}H_{18}O_2N_4Cl$: N = 19.31 per cent.

Found: N = 19.16 per cent.

Most of the substance was recovered unchanged after heating in a pressure bottle at 100° with hexamethylenetetramine in dry chloroform for several hours.

The following two substances were encountered in the course of experiments which were not carried to completion:

p-Diethylaminobenzeneazo- β -naphthylamine.—18 grams of *p*-aminodiethylaniline were diazotized in the usual manner and the solution was poured slowly into a well stirred solution of 15.5 grams of β -naphthylamine in about 150 cc. of warm 90 per cent alcohol. A deep red color developed at once. After about fifteen minutes, sodium acetate was added in excess, precipitating a gummy mass which solid-

ified on standing over night. 9.3 grams were obtained after recrystallization from 95 per cent alcohol. After another recrystallization, the compound forms glistening, maroon plates melting at $117-20^{\circ}$ with preliminary softening. When pulverized, it forms a brick-red powder, readily soluble in benzene and chloroform and dissolves in dilute acids to form deep red solutions which dye silk a brown-orange color.

0.1208 gm. of substance gave 19.65 cc. N (763 mm. and 23.5°).

Calculated for $C_{20}H_{22}N_4$: N = 17.60 per cent.

Found: N = 18.27 per cent.

p - Diethylaminobenzeneazochloroacetyl - α - naphthylamine.—11 grams of *p*-aminodiethylaniline were diazotized and the solution was slowly added to a well stirred solution of 10 grams of α -naphthylamine in warm 90 per cent alcohol. Stirring was continued for one hour, after which sodium acetate was added, precipitating a resinous mass which eventually became of such consistency that it could be filtered off and dried. As the amine could not be obtained in a crystalline state, it was dissolved in benzene and treated with one molecular equivalent of chloroacetic anhydride, the solution being eventually warmed. On cooling, a gum separated. This solidified on long standing, after which it was pulverized, washed well with dilute alkali and water, dried, and recrystallized from amyl alcohol, yielding 7.8 grams of chocolate-brown micro-crystals. These darken and soften above 120° and melt at 145° to a thick tar.

0.1716 gm. of substance (Kjeldahl) required 17.35 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{22}H_{23}ON_2Cl$: N = 14.19 per cent.

Found: N = 14.16 per cent.

The hexamethylenetetraminium salt was obtained only in an impure condition.

o-Chloroacetylaminophenol was obtained in practically quantitative yield by dissolving 4 grams of *o*-aminophenol in boiling benzene, adding 7 grams of chloroacetic anhydride, and boiling the resulting solution for fifteen minutes. The substance separated on cooling as a thick mass of needles and conformed to the description given by Aschan.²⁵

²⁵ O. Aschan: *Ber. d. deutsch. chem. Gesellsch.*, xx, p. 1524, 1887.

o-Chloroacetylaminophenol and Hexamethylenetetramine.—The components were boiled for fifteen minutes in dry chloroform, the clear solution first obtained being concentrated to small bulk at the same time. A precipitation of caseous material occurred, which rapidly became crystalline. The product was filtered off, ground up with dry chloroform, washed with dry acetone, and finally boiled one-half hour with dry acetone in order to remove a small amount of material insoluble in water. The salt melts with decomposition at 155° , dissolves in absolute alcohol, and is readily soluble in water, the solution giving a purple color with ferric chloride.

0.2676 gm. of substance gave 0.1194 gm. AgCl.

Calculated for $C_{14}H_{10}O_2N_2Cl$: Cl = 10.89 per cent.

Found: Cl = 11.04 per cent.

o-Chloroacetylaminophenyl Benzoate.—3 grams of benzoyl chloride were carefully added to a well chilled solution of 4 grams of *o*-chloroacetylaminophenol in 20 grams of pyridine. After fifteen minutes, the mixture was poured into water, precipitating an oil which solidified on scratching. The yield was practically quantitative. Recrystallized first from absolute, then 85 per cent alcohol, the benzoate forms silky needles melting at $107.5-8.5^{\circ}$ (corrected). It is very soluble in chloroform and benzene, rather difficultly in dry ether.

0.2041 gm. of substance (Kjeldahl) required 7.1 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{18}H_{12}O_2NCl$: N = 4.84 per cent.

Found: N = 4.87 per cent.

o-Chloroacetylaminophenyl Benzoate and Hexamethylenetetramine.—The clear solution obtained on boiling the components in dry chloroform was evaporated to small bulk and treated with dry acetone. The precipitated salt, which retained chloroform of crystallization, even on drying to constant weight *in vacuo* at the temperature of boiling alcohol over sulphuric acid, was boiled with dry acetone. As a result, acetone of crystallization was substituted for the chloroform and was held with equal tenacity. The presence of the acetone was proven by the iodoform test.

0.2035 gm. of substance required 8.12 cc. AgNO₃ Solution II.

Calculated for $C_{21}H_{15}O_3N_2Cl \cdot CH_3COCH_3$: Cl = 7.27 per cent.

Found: Cl = 7.42 per cent.

o-Chloroacetylaminophenyl *p*-Nitrobenzoate.—2.3 grams of *o*-chloroacetylaminophenol were dissolved in 15 grams of pyridine, cooled in a freezing mixture, and treated with a solution of 2.3 grams of *p*-nitrobenzoyl chloride in a little chloroform. After ten minutes at room temperature, the mixture was poured into dilute sulphuric acid and the chloroform removed by a current of air. The ester was filtered off and dried. Yield: practically quantitative. Recrystallized first from absolute alcohol, then toluene, it forms delicate, interlaced needles which soften at about 160° and melt at $163.5-4^{\circ}$ (corrected). It is difficultly soluble in cold absolute alcohol, benzene, and toluene, readily in chloroform.

0.1450 gm. of substance (Kjeldahl) required 8.45 cc. $\frac{N}{10}$ HCl.

0.1685 gm. of substance gave 0.0713 gm. AgCl.⁷

Calculated for $C_{15}H_{11}O_5N_2Cl$: N = 8.37 per cent; Cl = 10.59 per cent.

Found: N = 8.16 per cent; Cl = 10.47 per cent.

o-Chloroacetylaminophenyl *p*-Nitrobenzoate and Hexamethylene-tetramine.—To the clear solution obtained by boiling the components in a small volume of dry chloroform were added several volumes of dry acetone. After crystallization had been induced by scratching, the mixture was allowed to stand over night. The salt forms pale yellow plates which darken at about 166° and melt at $171-2^{\circ}$.

0.1798 gm. of substance required 4.05 cc. AgNO₃ Solution I.

Calculated for $C_{21}H_{23}O_5N_6Cl$: Cl = 7.46 per cent.

Found: Cl = 7.92 per cent.

m-Chloroacetylaminophenol.—8.5 grams of chloroacetic anhydride were added to a solution of 5 grams of *m*-aminophenol in boiling benzene. The solution was boiled for three-quarters of an hour and filtered from a small amount of tar which had deposited. On cooling, a tar first separated, followed by a crystalline deposit which was increased by adding ligroin. Recrystallized from water, the compound forms almost colorless, glistening plates melting at $134.5-6^{\circ}$ (corrected). Yield: 3.8 grams. It is easily soluble in dilute ammonia and sodium carbonate solutions and dissolves with difficulty in chloroform.

0.2157 gm. of substance (Kjeldahl) required 11.4 cc. $\frac{N}{10}$ HCl.

Calculated for $C_9H_9O_2NCl$: N = 7.55 per cent.

Found: N = 7.40 per cent.

m-Chloroacetylaminophenol and Hexamethylenetetramine.—

When a solution of the components in dry chloroform was boiled, a gummy mass soon separated. After about fifteen minutes' boiling, dry acetone was added, causing partial crystallization. After several days, during which most of the product had crystallized, the salt was filtered off, boiled about fifteen minutes with a little dry chloroform, and finally with dry acetone. When dried to constant weight *in vacuo* over calcium chloride and paraffin at room temperature, the salt retained acetone of crystallization. This was shown by the analysis and by the strong iodoform test given by the distillate from the aqueous solution. The salt decomposes at 168° with preliminary darkening. It is easily soluble in water, the solution giving a violet color with ferric chloride.

0.2657 gm. of substance gave 0.1067 gm. AgCl.

Calculated for $C_{14}H_{12}O_2N_2Cl \cdot \frac{1}{2}CH_3COCH_3$: Cl = 10.00 per cent.

Found: Cl = 9.94 per cent.

Benzeneazo-m-chloroacetylaminophenol.—A solution of 1.5 grams of aniline, diazotized in the usual way, was added to a well stirred solution of 2.8 grams of *m*-chloroacetylaminophenol in about 400 cc. of ice water containing 2.4 grams of sodium hydroxide. After fifteen minutes, the dye was salted out from the dark red-brown solution, ground up to a paste with ice water, stirred well, and treated with a slight excess of dilute sulphuric acid. The product was filtered off, washed well with cold water, and recrystallized twice from absolute alcohol, forming minute, golden, interlaced needles. Yield: 3 grams. The compound melts at 200° (corrected) with decomposition. It is soluble in sulphuric acid with a brown-orange color and gives a yellow solution with dilute sodium hydroxide which dyes silk a very pale yellow shade.

0.1095 gm. of substance gave 14.0 cc. N (765 mm. and 23°).

Calculated for $C_{14}H_{12}O_2N_2Cl$: N = 14.51 per cent.

Found: N = 14.44 per cent.

Benzeneazo-m-chloroacetylaminophenol and Hexamethylenetetramine.—As the insolubility of the dye in chloroform prevented the use of this solvent, the components were heated in a sealed tube with dry acetone at 100° for about one and one-half hours. The crude

product was ground up and boiled out with dry acetone. The yellow salt thus obtained decomposes from 202 to 230°. It is insoluble in water, but readily gives a clear, orange solution with a little dilute sodium hydroxide, dyeing silk a pale yellow shade.

0.2835 gm. of substance (Carius) gave 0.0895 gm. AgCl.

Calculated for $C_{20}H_{24}O_2N_7Cl$: Cl = 8.25 per cent.

Found: Cl = 7.81 per cent.

p-Chloroacetylaminophenyl Chloroacetate.—This substance was obtained in an attempt to prepare *p*-chloroacetylaminophenol by the pyridine method. On pouring the reaction mixture into iced 25 per cent sulphuric acid, only a small amount of precipitate was obtained. This was washed well with water and recrystallized first from absolute alcohol, then toluene, forming practically colorless spears softening at 168° and melting at 170–1° (corrected). The substance is insoluble in dilute acids or alkalies and is saponified by warming with dilute sodium hydroxide.

0.2062 gm. of substance (Kjeldahl) required 7.90 cc. $\frac{N}{10}$ HCl.

0.2769 gm. of substance gave 0.3026 gm. AgCl.⁷

Calculated for $C_{10}H_9O_3NCl_2$: N = 5.35 per cent; Cl = 27.06 per cent.

Found: N = 5.37 per cent; Cl = 27.04 per cent.

Chloroacetyl-*o*-anisidine.—In spite of the fact that this substance was used by Majert²⁶ and Frerichs⁶ we were unable to find a description of it in the literature. We prepared it in the usual manner from *o*-anisidine in benzene solution, aqueous sodium hydroxide, and chloroacetyl chloride. The benzene layer was shaken out successively with dilute hydrochloric acid, water, sodium carbonate solution, and water, and finally dried and evaporated to small bulk *in vacuo*. The residue crystallized on chilling and rubbing, and, after two recrystallizations from absolute alcohol with the aid of a freezing mixture, formed flat needles melting at 48–8.5° (corrected) with slight preliminary softening. It is readily soluble at room temperature in the usual organic solvents except ligroin, and gives a faint pink color with sulphuric acid.

0.3329 gm. of substance (Kjeldahl) required 16.8 cc. $\frac{N}{10}$ HCl.

Calculated for $C_9H_9O_2NCl$: N = 7.02 per cent.

Found: N = 7.07 per cent.

²⁶ D. R. P. 59121; Friedlaenders *Fortschr. d. Teerfarbenfabrikation*, iii, p. 915, 1890–94.

Chloroacetyl-o-anisidine and Hexamethylenetetramine.—The solution obtained by boiling the components in dry chloroform was evaporated to small bulk. The salt separated out in almost quantitative yield after the addition of dry ether. It melts at $151-3^{\circ}$, is readily soluble in water, and dissolves in sulphuric acid with a violet-rose color.

0.2247 gm. of substance required 6.65 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{15}\text{H}_{17}\text{O}_2\text{N}_6\text{Cl}$: Cl = 10.44 per cent.

Found: Cl = 10.42 per cent.

α -Iodopropionyl-*o*-anisidine.—11.5 grams of α -iodopropionyl chloride²⁷ were added, drop by drop, to a solution of 16 grams of *o*-anisidine in alcohol-free ether, with cooling and stirring. After warming for a few minutes, the mixture was allowed to stand in the ice box for several days. The *o*-anisidine hydrochloride was filtered off and the filtrate shaken out successively with dilute hydrochloric acid containing a little sodium bisulphite, with water, dilute sodium carbonate solution, and water. The ether solution was then dried over calcium chloride, evaporated to dryness, and the residue recrystallized from ligroin, forming pale cream-colored, felted needles. Yield: 14.9 grams. It is readily soluble in the usual organic solvents. Recrystallized from dry, alcohol-free ether, it forms delicate needles melting at $95-6.5^{\circ}$ (corrected) with slight preliminary softening.

0.3119 gm. of substance (Kjeldahl) required 10.2 cc. $\frac{N}{10}$ HCl.

Calculated for $\text{C}_{10}\text{H}_{12}\text{O}_2\text{NI}$: N = 4.59 per cent.

Found: N = 4.58 per cent.

When heated with hexamethylenetetramine in dry chloroform or dry acetone, the substance yielded, in addition to unchanged materials, only hexamethylenetetraminium iodide.

β -Iodopropionyl Chloride.—This was prepared essentially according to Abderhalden and Gressel,²⁸ who, however, did not describe their product. The chloride boils at $80.5-1.5^{\circ}$ at 15 mm., and, after shaking with mercury to remove a small amount of dissolved iodine, forms a practically colorless, heavy, strong smelling oil.

²⁷ E. Abderhalden and M. Guggenheim: *Ber. d. deutsch. chem. Gesellsch.*, xli, p. 2855, 1908.

²⁸ E. Abderhalden and E. Gressel: *Ztschr. f. physiol. Chem.*, lxxiv, p. 476, 1911.

0.1702 gm. of substance required 7.88 cc. AgNO_3 Solution I.²⁹

Calculated for $\text{C}_9\text{H}_9\text{OCl}$: Cl = 16.24 per cent.

Found: Cl = 16.30 per cent.

β -Iodopropionyl-o-anisidine.—This was prepared in the same way as the α -iodo analog. The reaction mixture was well shaken and allowed to stand about two hours before filtering off the hydrochloride of the amine. It was found necessary to use a large volume of ether in order to prevent the acyl derivative from crystallizing out with the hydrochloride. Yield: 19.4 grams from 19 grams of *o*-anisidine and 15 grams of β -iodopropionyl chloride, after recrystallization from dilute alcohol. Recrystallized first from methyl alcohol and then benzene, the compound forms colorless, irregular plates which soften at 88° and melt at $89\text{--}91^\circ$ (corrected). It is much less soluble than the α -isomer.

0.3113 gm. of substance (Kjeldahl) required 10.1 cc. $\frac{N}{16}$ HCl.

Calculated for $\text{C}_{10}\text{H}_{12}\text{O}_2\text{NI}$: N = 4.59 per cent.

Found: N = 4.54 per cent.

β -Iodopropionyl-o-anisidine and Hexamethylenetetramine.—A solution of equimolecular amounts of the components in dry chloroform was heated to the boiling point, whereupon the salt started to separate in thin, glistening, micro-platelets. After boiling for fifteen minutes, the salt was filtered off. Additional quantities were obtained by longer boiling of the filtrate. The salt melts at $172\text{--}3^\circ$ and is very difficultly soluble in water.

0.2237 gm. of substance required 5.15 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{16}\text{H}_{24}\text{O}_2\text{N}_6\text{I}$: I = 28.51 per cent.

Found: I = 28.98 per cent.

Chloroacetyl- ω -o-anisidinoacetophenone.—7.5 grams of phenacyl-*o*-anisidine³⁰ were dissolved in benzene and 2.9 cc. of chloroacetyl chloride slowly added, with cooling and shaking. The mixture, which had turned green and contained a curdy precipitate, was warmed about three hours on the water bath, hydrochloric acid being evolved. After cooling, the solution was filtered and treated with several volumes of ligroin. An oil precipitated, which soon solidified and was recrystallized from dilute alcohol, with bone-

²⁹ Hydrolysis with cold, dilute, aqueous ammonia.

³⁰ M. Busch and G. Hefele: *Jour. f. prakt. Chem.*, lxxxiii, p. 442, 1911.

blackening. In this way were obtained 7.7 grams of prisms. Recrystallized from methyl alcohol, the substance forms prismatic needles melting at $101.5-2.5^{\circ}$ (corrected). It is soluble in benzene, difficultly in cold methyl alcohol and ether.

0.3305 gm. of substance (Kjeldahl) required 10.15 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{17}H_{16}O_2NCl$: N = 4.42 per cent.

Found: N = 4.30 per cent.

*Chloroacetyl- ω -*o*-anisidinoacetophenone and Hexamethylenetetramine.*—After the chloroform solution of the components had been boiled for about twelve hours, the small amount of precipitate was filtered off and the solution allowed to stand. The salt slowly separated in poor yield in the form of colorless micro-prisms. It melts at 145° with decomposition and is readily soluble in water and alcohol. The solution in sulphuric acid has a pale brownish yellow color.

0.2501 gm. of substance required 5.73 cc. $AgNO_3$ Solution I.

Calculated for $C_{22}H_{20}O_2N_2Cl$: Cl = 7.75 per cent.

Found: Cl = 8.06 per cent.

*Chloroacetyl-*p*-anisidine.*—This substance was also used by Majert²⁶ and Frerichs,⁶ but was not described. It was prepared by us in the usual way from *p*-anisidine in benzene solution, aqueous sodium hydroxide, and chloroacetyl chloride. Ligroin was added to the benzene layer and the solution allowed to stand in the cold over night. The deposited substance was then recrystallized twice from absolute alcohol, forming glistening, transparent prisms melting at $121.5-2.5^{\circ}$ (corrected) with preliminary softening. It is difficultly soluble in the cold in benzene, fairly readily in ether, chloroform, and alcohol. It gives no color in sulphuric acid.

0.3091 gm. of substance (Kjeldahl) required 15.02 cc. $\frac{N}{10}$ HCl.

Calculated for $C_9H_{10}O_2NCl$: N = 7.02 per cent.

Found: N = 6.81 per cent.

*Chloroacetyl-*p*-anisidine and Hexamethylenetetramine.*—The salt melts at $164-7^{\circ}$ with decomposition. It dissolves readily in water and gives a pale brownish yellow solution with sulphuric acid.

0.1971 gm. of substance required 10.73 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{12}\text{H}_{12}\text{O}_2\text{N}_2\text{Cl}$: Cl = 10.44 per cent.

Found: Cl = 10.13 per cent.

o-Chloroacetylaminobenzyl Alcohol.—12.3 grams of *o*-aminobenzyl alcohol were dissolved in dry, alcohol-free ether, well cooled, and treated with a solution of 5.5 grams of chloroacetyl chloride in dry benzene. The mixture was treated with water to dissolve the precipitated hydrochloride, dried, and concentrated to small bulk. The residue, which crystallized on cooling, was recrystallized from a small volume of benzene with the aid of ligroin. Yield: 7 grams. After two recrystallizations from benzene, the substance forms rosettes of needles melting at 103° (corrected).

0.2000 gm. of substance (Kjeldahl) required 9.64 cc. $\frac{N}{10}$ HCl.

Calculated for $\text{C}_9\text{H}_{10}\text{O}_2\text{NCl}$: N = 7.02 per cent.

Found: N = 6.76 per cent.

o-Chloroacetylaminobenzyl Alcohol and Hexamethylenetetramine.—The boiling chloroform solution of the components soon deposited an oil which gradually crystallized and set to a solid cake. This was disintegrated and boiled with a little more dry chloroform for about half an hour. As the product thus obtained contained chloroform of crystallization, it was boiled for one hour with dry acetone. The salt melts with decomposition at $144-5^\circ$. It is somewhat hygroscopic and is very soluble in water.

0.2245 gm. of substance required 6.9 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{12}\text{H}_{12}\text{O}_2\text{N}_2\text{Cl}$: Cl = 10.44 per cent.

Found: Cl = 10.81 per cent.

o-Chloroacetylaminobenzyl Benzoate.—The ester was prepared in the usual way from 5 grams of *o*-chloroacetylaminobenzyl alcohol in pyridine and 4 grams of benzoyl chloride. The crude product was washed with dilute sodium carbonate solution and recrystallized from absolute alcohol. Yield: 3.9 grams. Recrystallized first from absolute alcohol and then toluene, the ester forms snow-white needles melting at $113-3.5^\circ$ (corrected), with slight preliminary softening. It dissolves readily in ether and benzene.

0.1792 gm. of substance required 11.00 cc. AgNO_3 Solution II.⁷

Calculated for $\text{C}_{18}\text{H}_{14}\text{O}_4\text{NCl}$: Cl = 11.68 per cent.

Found: Cl = 11.42 per cent.

o-Chloroacetylaminobenzyl Benzoate and Hexamethylenetetramine.—The salt crystallized out on adding dry acetone to the chloroform solution and scratching. It darkens above 140° and melts at $154-5^{\circ}$ to a thick, greenish liquid.

0.1989 gm. of substance required 8.35 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{22}\text{H}_{20}\text{O}_3\text{N}_2\text{Cl}$: Cl = 7.99 per cent.

Found: Cl = 7.81 per cent.

p-Chloroacetylaminobenzoic Acid Ethyl Ester.—This was prepared in the usual way from the amino ester in toluene, chloroacetyl chloride, and normal potassium hydroxide solution. The substance conformed to the description given by Einhorn.³¹

p-Chloroacetylaminobenzoic Acid Ethyl Ester and Hexamethylenetetramine.—The salt retained chloroform, which was only very slowly given off *in vacuo* at the temperature of boiling alcohol. It is very soluble in water and melts at $155-6^{\circ}$ with decomposition.

0.2264 gm. of substance required 5.68 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{17}\text{H}_{20}\text{O}_3\text{N}_2\text{Cl}$: Cl = 9.29 per cent.

Found: Cl = 8.83 per cent.

p-Chloroacetylaminobenzoic Acid Diethylaminoethyl Ester (Chloroacetyl novocain).—28 grams of novocain (hydrochloride) were treated with 210 cc. of normal sodium hydroxide solution and 50 cc. of benzene added to redissolve the free base. To the mixture were added, drop by drop and with cooling and shaking, 20 grams of chloroacetyl chloride. The mixture was made distinctly alkaline with sodium hydroxide and shaken out with ether. The upper layer was dried and concentrated until the ether had distilled off. On adding ligroin cautiously to the benzene solution, this set to a thick mass of microscopic needles. These were filtered off and washed with ligroin. Yield: 21 grams. For analysis, the substance was recrystallized from ligroin and then melted at $63-4.5^{\circ}$ (corrected). It does not keep well in the crude state.

0.2078 gm. of substance required 6.7 cc. AgNO_3 Solution I.⁷

Calculated for $\text{C}_{18}\text{H}_{22}\text{O}_3\text{N}_2\text{Cl}$: Cl = 11.34 per cent.

Found: Cl = 11.35 per cent.

³¹ D. R. P. 106502; *Friedlaenders Fortschr. d. Teerfarbenfabrikation*, v, p. 820, 1897-1900.

p-Chloroacetylaminobenzoic Acid Diethylaminoethyl Ester and Hexamethylenetetramine.—To the clear chloroform solution were added several volumes of dry acetone. On cooling, the solution set to a thick mass. This was filtered off, washed with acetone, and recrystallized by dissolving in a little chloroform and precipitating with dry acetone. It was not found possible to further purify the salt. It forms lenticular micro-platelets which sinter at 126° and melt at 128–9°.

0.0768 gm. of substance (Kjeldahl) required 9.9 cc. $\frac{N}{10}$ HCl.

0.1744 gm. of substance required 8.76 cc. AgNO₃ Solution II.

Calculated for C₂₁H₂₈O₃N₄Cl: N = 18.55 per cent; Cl = 7.84 per cent.

Found: N = 18.05 per cent; Cl = 9.34 per cent.

m-Chloroacetylaminacetophenone.—21.5 grams of *m*-aminoacetophenone were chloroacetylated in the usual manner, the mixture being allowed to become warm in order to ensure complete solution of the amine. After cooling, the chloroacetyl derivative was filtered off and washed with water and toluene. After bone-blackening in benzene, the product was obtained by addition of ligroin to the hot, filtered solution. Yield: 23.5 grams. Recrystallized from absolute alcohol, the substance forms prisms melting at 113.5–14.5° (corrected). It is rather difficultly soluble in cold absolute alcohol, benzene, and ether, more readily in chloroform and acetone. It turns a pale greenish yellow in sulphuric acid and dissolves with a very faint color.

0.3154 gm. of substance (Kjeldahl) required 14.90 cc. $\frac{N}{10}$ HCl.

Calculated for C₁₀H₁₀O₂NCl: N = 6.62 per cent.

Found: N = 6.61 per cent.

m-Chloroacetylaminacetophenone and Hexamethylenetetramine.—The salt forms micro-platelets which melt with decomposition at 165–6°. It is rather difficultly soluble in water. With absolute alcohol, it changes to a mass of hair-like needles. Sulphuric acid turns the salt yellow and dissolves it with a pale greenish yellow color.

0.2183 gm. of substance required 6.12 cc. AgNO₃ Solution I.

Calculated for C₁₆H₂₂O₃N₄Cl: Cl = 10.08 per cent.

Found: Cl = 9.94 per cent.

p-Chloroacetylaminoleucomalachite Green.—5 grams of amino compound were chloroacetylated in benzene solution in the presence of an excess of two-normal sodium hydroxide solution. It was found convenient to carry out the operation in a separatory funnel, owing to the vigorous shaking required for the decomposition of the difficultly soluble addition products first formed upon addition of the chloride to the mixture. The benzene solution was washed with water, dried over sodium sulphate and evaporated to small bulk. A portion of the dark green syrup thus obtained was taken up in alcohol, water added until slightly turbid, and allowed to stand, with occasional rubbing. The pale greenish micro-crystals finally deposited were recrystallized from absolute alcohol with the aid of a freezing mixture. The compound sinters above 80° and gradually softens to a tar when rapidly heated.

0.1592 gm. of substance (Carius) gave 0.0539 gm. AgCl.

Calculated for $C_{22}H_{22}ON_3Cl$: Cl = 8.41 per cent.

Found: Cl = 8.37 per cent.

p-Chloroacetylaminoleucomalachite Green and Hexamethylenetetramine.—The crude, dark green syrup resulting on evaporation of the benzene layer obtained in the chloroacetylation of 5 grams of amino compound was taken up in 10 cc. of dry chloroform. 2 grams of hexamethylenetetramine were added and the mixture was boiled for one hour. Most of the chloroform was boiled off from the clear green solution and dry acetone added. The solution was poured off and the residue again treated with dry acetone. On standing over night, the salt separated from the acetone solutions as a nearly colorless powder. In subsequent preparations, it was merely necessary to add dry acetone to a concentrated chloroform solution of the salt, and seed the solution with a crystal from the first experiment. The salt melts at $189-90^{\circ}$ with decomposition, and is insoluble in water, dissolving readily, however, in dilute mineral acids. Yield: 5.6 grams.

0.2250 gm. of substance gave 0.0587 gm. AgCl.

Calculated for $C_{21}H_{20}ON_4Cl$: Cl = 6.31 per cent.

Found: Cl = 6.45 per cent.

o-Chloroacetyl-amino-*p'*, *p''*-Tetraethyldiaminotriphenylmethane

and Hexamethylenetetramine.—Starting with *o*-amino-*p'*, *p''*-tetraethyldiaminotriphenylmethane, the chloroacetyl derivative was obtained as a dark green syrup and was added to hexamethylenetetramine in the same way as in the case of the preceding substance. The chloroform solution was evaporated to dryness *in vacuo*, treated with dry acetone, filtered, and again evaporated *in vacuo*. On adding a mixture of ether and water, the colored impurities were taken up by the ether while the salt remained suspended in the aqueous layer in the form of colorless micro-platelets. These were filtered off, washed alternately with water and ether, and dried. The product melts at 177–8° with decomposition and dissolves readily in dilute mineral acids.

0.1739 gm. of substance required 2.8 cc. AgNO₃ Solution I.

Calculated for C₂₆H₂₄ON₂Cl: Cl = 5.74 per cent.

Found: Cl = 5.67 per cent.

p-Chloroacetyl-amino-*p'*, *p''*-Tetraethyldiaminotriphenylmethane and Hexamethylenetetramine.—The *p*-amino compound was chloroacetylated and the crude product added to hexamethylenetetramine in the same way as in the preceding examples. After boiling off the chloroform, several volumes of dry acetone were added, whereupon the salt crystallized out on scratching. The precipitate of micro-plates was filtered off and washed well with dry acetone, practically all the green color being removed in this way. The salt is insoluble in water, but dissolves readily in one molecular equivalent of dilute mineral acid. It melts at 168–9°. Yield: 5.5 grams, from 5 grams of amino compound.

0.2131 gm. of substance required 3.6 cc. AgNO₃ Solution I.

Calculated for C₂₆H₂₄ON₂Cl: Cl = 5.74 per cent.

Found: Cl = 5.95 per cent.

6-Chloroacetylaminquinoline.—12 grams of 6-aminoquinoline were chloroacetylated in the usual way in benzene solution with chloroacetyl chloride and an excess of normal sodium hydroxide solution. The substance separated as an oil, which soon solidified. This was filtered off, washed with water and benzene, and recrystallized, with bone-black, from a mixture of benzene and toluene. Yield: 9.9 grams of minute prisms which melt to a red tar at 153–5°

after turning red. The substance is very soluble in alcohol and is very irritating to the mucous membrane.

0.2022 gm. of substance gave 22.42 cc. N (767.5 mm. and 21.5°).

Calculated for $C_{11}H_9ON_2Cl$: N = 12.79 per cent.

Found: N = 12.69 per cent.

The *hydrochloride* was precipitated from the mother liquors from the recrystallization by passing in a stream of hydrochloric acid gas. It was recrystallized by dissolving in 85 per cent alcohol and adding acetone to the warm solution. The salt forms thin, pale red-brown platelets which turn brown above 220°, then darken and sinter, and finally decompose above 250°. It is very irritating to the mucous membrane and is easily soluble in water, yielding an orange solution.

0.1797 gm. of substance required 7.2 cc. $\frac{N}{10}$ NaOH for neutralization.

Calculated for $C_{11}H_9ON_2Cl \cdot HCl$: HCl = 14.19 per cent.

Found: HCl = 14.49 per cent.

6-Chloroacetylaminquinoline and Hexamethylenetetramine.—

The crude salt was washed well and ground up with dry chloroform and then treated in the same manner with dry acetone. It forms pale drab-colored micro-platelets which dissolve readily in water and melt with decomposition at 203–4°.

0.4316 gm. of substance required 11.63 cc. $AgNO_3$ Solution I.

Calculated for $C_{17}H_{12}ON_2Cl$: Cl = 9.83 per cent.

Found: Cl = 9.49 per cent.

THE QUATERNARY SALTS OF HEXAMETHYLENE- TETRAMINE.

IV. MONOHALOGENACYLATED SIMPLE AMINES, UREAS, AND URETHANES, AND THE HEXAMETHYLENETETRAMINIUM SALTS DERIVED THEREFROM.

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With but one exception, the hexamethylenetetraminium salts described in this paper are purely of aliphatic origin and were obtained from the halogenacyl compounds and hexamethylenetetramine by the methods described in the previous papers. The salts are all easily soluble in water. On standing, more rapidly on warming, the solutions decompose with the liberation of formaldehyde and other products, which, contrary to the behavior observed with the aromatic compounds, remain in solution. A search of the literature has revealed to us the description of the chloroacetyl derivative of but one simple aliphatic amine; *viz.*, chloroacetylallylamine.¹ Our work has necessitated the preparation of the chloroacetyl compounds of several of the simpler amines and the preparation and properties of these will be found below.

EXPERIMENTAL.

(1) *Monohalogenacyl Derivatives of Simple Amines.*

Chloroacetamide may be easily prepared in excellent yield as follows: Ethyl chloroacetate is well cooled in a freezing mixture and vigorously turbined. Two volumes of well cooled, concentrated aque-

¹ C. Harries and I. Petersen: *Ber. d. deutsch. chem. Gesellsch.*, xliii, pp. 635 and 1758, 1910.

ous ammonia are then added. The reaction occurs smoothly with the formation of a thick paste of the amide. After standing several hours at room temperature, the product is filtered off, washed, and dried, and then agrees in properties with those described in the literature.

Chloroacetamide and hexamethylenetetramine. This substance was first prepared by Einhorn and Göttler,² with whose description our preparation agreed.

α-Iodopropionamide.—27 cc. of concentrated aqueous ammonia were diluted to about 50 cc., placed in a freezing mixture, vigorously turbinated, and treated with 30 grams of *α*-iodopropionyl chloride,³ added drop by drop. After about half an hour's stirring, the amide was filtered off. In the crude state it turned slightly yellow, but lost its sensitiveness to light after recrystallization from water. Yield: 20.5 grams. Recrystallized from toluene, the amide forms long, glistening needles which soften and turn yellow above 150° and melt at 159–60.5° (corrected). It is difficultly soluble in benzene, toluene, and cold water. The aqueous solution, boiled with a little silver nitrate, gives a copious precipitate of silver iodide.

0.4165 gm. of substance (Kjeldahl) required 21.0 cc. $\frac{N}{10}$ HCl.

Calculated for C_3H_5ONI : N = 7.01 per cent.

Found: N = 7.06 per cent.

Under the conditions used, the amide did not yield a quaternary salt with hexamethylenetetramine.

β-Iodopropionamide. This was prepared from 30 grams of *β*-iodopropionyl chloride⁴ in the same way as the *α*-compound. In the crude state the amide rapidly turns pink, but loses its sensitiveness to light after bone-blackening and recrystallizing from water. Yield: 20 grams. According to Henry,⁵ who prepared the amide from methyl *β*-iodopropionate and aqueous ammonia, it melts at 100–1°. Our preparation was recrystallized from toluene, forming feathery plates which melted to a yellow liquid at 141.5–2.5° (cor-

² A. Einhorn and M. Göttler: *Ann. d. Chem.*, cccxi, p. 150, 1908.

³ E. Abderhalden and M. Guggenheim: *Ber. d. deutsch. chem. Gesellsch.*, xli, p. 2855, 1908.

⁴ E. Abderhalden and E. Gressel: *Ztschr. f. physiol. Chem.*, lxxiv, p. 476, 1911, and Paper III of this series (this *Journal*, xxi, p. 103, 1915).

⁵ L. Henry: *Compt. rend. Acad. d. sc.*, c, p. 117, 1885.

rected) with preliminary softening. It is difficultly soluble in benzene and toluene and is fairly soluble in cold water.

0.3611 gm. of substance (Kjeldahl) required 18.50 cc. $\frac{N}{10}$ HCl.

Calculated for $C_6H_8ON_2$: N = 7.01 per cent.

Found: N = 7.17 per cent.

β -Iodopropionamide and Hexamethylenetetramine.—12 grams of amide and 8.5 grams of base are dissolved in about 750 cc. of commercial acetone, which is then boiled gently for about three hours in a water bath heated to 60–70°. After a short time, the salt begins to separate in the form of broad, glistening needles, and care must be taken to keep within the limits of temperature given in order to avoid superheating on the bottom and sides of the flask and consequent decomposition of the heavy precipitate. Yield: 16.1 grams. The salt melts with decomposition at 169–70°.

0.1769 gm. of substance required 5.28 cc. $AgNO_3$ Solution I.⁶

Calculated for $C_6H_{12}ON_4I$: I = 37.43 per cent.

Found: I = 37.58 per cent.

Chloroacetmethylamide.—13 grams of methylamine hydrochloride (in aqueous solution) were well cooled and treated with 190 cc. of 10 per cent sodium hydroxide solution. The solution was then placed in a freezing mixture, well turbined, and treated, drop by drop, with 23 grams of chloroacetyl chloride. After making slightly acid with hydrochloric acid, the mixture was extracted twelve times with chloroform. This was dried over calcium chloride, evaporated to small bulk, diluted with ligroin, and placed in a freezing mixture. The amide crystallized out and was filtered off in the cold. Yield: 9.3 grams. Recrystallized from dry, alcohol-free ether with the aid of a freezing mixture, the compound forms delicate needles melting at 45–6° (corrected) with preliminary softening. It is easily soluble at room temperature in water and the other usual solvents. When exposed to the air, it slowly volatilizes.

0.2040 gm. of substance (Kjeldahl) required 18.90 cc. $\frac{N}{10}$ HCl.

Calculated for C_5H_8ONCl : N = 13.03 per cent.

Found: N = 12.97 per cent.

⁶ 1 cc. = 0.00352 gm. Cl; 0.01259 gm. I.

Chloroacetmethylamide and Hexamethylenetetramine.—The salt gradually precipitates on boiling a solution of the components in dry chloroform. When heated, it turns brown and melts with decomposition at $173-4^{\circ}$. It is very soluble in water.

0.2141 gm. of substance required 8.60 cc. AgNO_3 Solution I.

Calculated for $\text{C}_5\text{H}_{12}\text{ON}_2\text{Cl}$: $\text{Cl} = 14.32$ per cent.

Found: $\text{Cl} = 14.14$ per cent.

Chloroacetdimethylamide.—30 grams of 33 per cent aqueous dimethylamine solution were diluted with an equal volume of water, cooled in a freezing mixture, and treated with 230 cc. of double normal sodium hydroxide solution. 19.6 cc. of chloroacetyl chloride (1.1 mols.) were then added, drop by drop, with vigorous shaking. At the end, the solution was made slightly acid with acetic acid and shaken out six times with ether. This was dried over calcium chloride, evaporated to small bulk, and the residue fractionated *in vacuo*. Yield: 6.6 grams, boiling at 11 mm. at $98.5-9.5^{\circ}$ (corrected, thermometer in vapor, bath at $130-5^{\circ}$). The amide crystallizes in a freezing mixture and melts at 15.5° (corrected). It is miscible in all proportions with water and alcohol and is very irritating to the skin.

0.2437 gm. of substance⁷ required 37.36 cc. AgNO_3 Solution II.⁸

0.5079 gm. (Kjeldahl) required 41.20 cc. $\frac{N}{10}$ HCl .

Calculated for $\text{C}_4\text{H}_8\text{ONCl}$: $\text{N} = 11.53$ per cent; $\text{Cl} = 29.17$ per cent.

Found: $\text{N} = 11.37$ per cent; $\text{Cl} = 28.52$ per cent.

Chloroacetdimethylamide and Hexamethylenetetramine.—The clear solution obtained by dissolving the components in dry chloroform and boiling for one hour was evaporated to small bulk. The addition of several volumes of dry acetone resulted in the precipitation of a gum which crystallized on rubbing. The salt was pulverized, boiled with dry acetone, and dried. It melts at $160-2^{\circ}$ to an orange liquid and is extremely soluble in water. It also dissolves in chloroform and alcohol.

0.2148 gm. of substance required 15.30 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{10}\text{H}_{20}\text{ON}_2\text{Cl}$: $\text{Cl} = 13.55$ per cent.

Found: $\text{Cl} = 13.25$ per cent.

⁷ Hydrolysis with alcoholic sodium hydroxide solution.

⁸ 1 cc. = 0.00186 gm. Cl .

Chloroacetethylamide.—This was prepared in the same way as the dimethyl isomer. 100 grams of 33 per cent aqueous ethylamine solution yielded 71 grams of amide, boiling at $97.5-8.5^{\circ}$ at 13 mm. and melting at $22-2.5^{\circ}$ (corrected).

0.0830 gm. of substance (Kjeldahl) required 6.9 cc. $\frac{N}{10}$ HCl.

Calculated for C_4H_9ONCl : N = 11.53 per cent.

Found: N = 11.64 per cent.

Chloroacetethylamide and Hexamethylenetetramine.—The salt separates slowly from a boiling solution of the components in dry chloroform as thick, hexagonal plates. It darkens above 160° and melts with decomposition at $167-8^{\circ}$. It is very soluble in water.

0.2037 gm. of substance required 7.75 cc. $AgNO_3$ Solution I.

Calculated for $C_{10}H_{20}ON_6Cl$: Cl = 13.55 per cent.

Found: Cl = 13.39 per cent.

Chloroacetdiethylamide.—The only variation from the procedure employed in the previous cases was that the reaction mixture was extracted ten times with chloroform. 15.2 grams of diethylamine yielded 11 grams of highly refracting liquid boiling at $125.5-30.5^{\circ}$ at 21–2 mm., of which most boiled at $126.5-8.5^{\circ}$ at 21 mm. It is easily soluble in water but not miscible with it in all proportions. It has a piquant odor which is pleasant at first but soon irritates the mucous membrane.

0.1382 gm. of substance (Kjeldahl) required 9.5 cc. $\frac{N}{10}$ HCl.

Calculated for $C_6H_{12}ONCl$: N = 9.37 per cent.

Found: N = 9.82 per cent.

Chloroacetdiethylamide and Hexamethylenetetramine.—The solution of the components in dry chloroform was boiled one hour, filtered from a small amount of what was probably hexamethylenetetraminium chloride, and concentrated to small volume. About four volumes of dry acetone were then added, causing the gradual deposition of the salt in glistening rhombs. These were pulverized and boiled with dry acetone. The salt melts at 174° to a turbid liquid which, on further heating, clears up, darkens, and finally effervesces above 190° . It is very soluble in water and also dissolves in chloroform and alcohol.

0.2020 gm. of substance required 7.07 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{12}\text{H}_{24}\text{ON}_4\text{Cl}$: $\text{Cl} = 12.27$ per cent.

Found: $\text{Cl} = 12.32$ per cent.

Chloroacetyl piperidide.—25 grams of piperidine were dissolved in about 150 cc. of dry, alcohol-free ether and cooled with ice water. To the solution was added, drop by drop, with continued cooling and shaking, a solution of 12 cc. of chloroacetyl chloride in dry, alcohol-free ether. After standing a short time, the mixture was shaken successively with small amounts of water, dilute hydrochloric acid, and dilute sodium carbonate solution. It is probable that the chief loss occurred at this point. The ethereal layer was dried over sodium sulphate, evaporated to small bulk, and fractionated *in vacuo*. After two distillations, the substance was obtained as a moderately viscous, practically colorless oil boiling at 12.5 mm. at $143.5\text{--}5.5^\circ$ (corrected, thermometer in vapor, bath at $155\text{--}75^\circ$), of which the greater part boiled at 144° . Yield: 8.7 grams. It is irritating to the mucous membrane and is soluble in cold water.

0.1924 gm. of substance gave 16.25 cc. N (753 mm. and 26.7°).

Calculated for $\text{C}_7\text{H}_{12}\text{ONCl}$: $\text{N} = 8.67$ per cent.

Found: $\text{N} = 9.15$ per cent.

Chloroacetyl piperidide and Hexamethylenetetramine.—The viscous solution obtained by boiling the components for two hours in a little dry chloroform was filtered, after which it set to a crystalline mass. On adding dry acetone, almost all the salt went into solution, precipitating out again almost immediately. The product was filtered off and boiled for one hour with dry acetone to remove chloroform of crystallization. The salt forms micro-platelets which dissolve easily in water. It becomes pasty at $168\text{--}70^\circ$ and melts completely at 184° with gas evolution.

0.1951 gm. of substance required 11.88 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{12}\text{H}_{24}\text{ON}_4\text{Cl}$: $\text{Cl} = 11.75$ per cent.

Found: $\text{Cl} = 11.33$ per cent.

Methylenebisiodoacetamide.—This was prepared from methylenebischloroacetamide⁹ and sodium iodide in dry acetone. On warming the mixture for a short time sodium chloride precipitated and

⁹ A. Einhorn and T. Mauermayer: *Ann. d. Chem.*, cccxliii, p. 284, 1905.

the new compound separated out. Water was added and the precipitate filtered off and recrystallized twice from acetic acid. It forms arborescent masses of thin plates which darken above 220° when rapidly heated, and decompose at $227-9^{\circ}$ with evolution of iodine.

0.2215 gm. of substance (Kjeldahl) required 12.05 cc. $\frac{N}{10}$ HCl.

Calculated for $C_6H_8O_2N_2I_2$: N = 7.34 per cent.

Found: N = 7.62 per cent.

The hexamethylenetetramine addition product of the compound could not be obtained in a state of purity.

Ethylenebischloroacetamide.—15 grams of ethylenediamine hydrochloride were dissolved in water and chloroacetylated in the usual manner with chloroacetyl chloride and sodium hydroxide. The product separated immediately in a state of purity. Yield: 14 grams. Recrystallized first from 85 per cent alcohol, then acetic acid, the substance forms prismatic needles melting at $174-6^{\circ}$ (corrected) with preliminary softening. It is difficultly soluble in the cold in the usual solvents.

0.1219 gm. of substance gave 14.15 cc. N (768 mm. and 22.4°).

Calculated for $C_6H_{10}O_2N_2Cl_2$: N = 13.15 per cent.

Found: N = 13.19 per cent.

(2) *Chloroacetylureas and Urethanes.*

Chloroacetylurea and Hexamethylenetetramine.—Equimolecular amounts of the components were boiled in acetone for four hours. The salt gradually separated in the form of plates which melted with decomposition at $176-7^{\circ}$ when freshly prepared, but showed a melting point of $191-2^{\circ}$ after standing for some weeks. It is very easily soluble in water.

0.1992 gm. of substance required 7.20 cc. $AgNO_3$ Solution I.

Calculated for $C_6H_{12}O_2N_4Cl$: Cl = 12.82 per cent.

Found: Cl = 12.72 per cent.

*Chloroacetylmethylurea*¹⁰ and *Hexamethylenetetramine*.—Equimolecular amounts of the components were dissolved separately in

¹⁰ G. Frerichs: *Chem. Zentralbl.*, lxx, pt. ii, p. 285, 1899.

hot acetone and the solutions mixed. The salt soon began to separate in long needles which were filtered off after forty-eight hours. It sinters at $149-51^{\circ}$ and gradually melts, decomposing at 178° . It is very soluble in water.

0.2129 gm. of substance required 7.1 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{10}\text{H}_{10}\text{O}_2\text{N}_4\text{Cl}$: $\text{Cl} = 12.20$ per cent.

Found: $\text{Cl} = 11.74$ per cent.

Chloroacetylbenzylurea.—One mol. of benzylurea was suspended in dry benzene and 1.1 mols. of chloroacetyl chloride were added. The mixture was boiled until the evolution of hydrochloric acid ceased, after which the chloroacetyl derivative crystallized out on cooling. Recrystallized from absolute alcohol, it forms glistening needles which melt at $153-5^{\circ}$ (corrected). It is difficultly soluble in the cold in absolute alcohol, benzene, chloroform, and acetone.

0.2571 gm. of substance (Kjeldahl) required 22.65 cc. $\frac{N}{10}$ HCl .

Calculated for $\text{C}_{10}\text{H}_{11}\text{O}_2\text{N}_2\text{Cl}$: $\text{N} = 12.37$ per cent.

Found: $\text{N} = 12.34$ per cent.

Chloroacetylbenzylurea and Hexamethylenetetramine.—After a few minutes' boiling, the salt separated from the solution of the components in dry chloroform as a mass of long, silky needles. Heating was continued for about fifteen minutes, after which the product was filtered off, washed with chloroform, and boiled with acetone. It is readily soluble in water and melts at 160° .

0.2125 gm. of substance required 5.75 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{16}\text{H}_{20}\text{O}_2\text{N}_6\text{Cl}$: $\text{Cl} = 9.67$ per cent.

Found: $\text{Cl} = 9.53$ per cent.

*Chloroacetylurethane*¹⁰ and *Hexamethylenetetramine*.—A solution of the components in dry chloroform was boiled for one-half hour and allowed to stand over night. The salt thus obtained in almost quantitative yield melts at $166-7^{\circ}$ and is very soluble in water.

0.2355 gm. of substance required 7.75 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{17}\text{H}_{20}\text{O}_2\text{N}_6\text{Cl}$: $\text{Cl} = 11.60$ per cent.

Found: $\text{Cl} = 11.58$ per cent.

ON THE WALDEN REARRANGEMENT IN THE HEXOSES.

BY P. A. LEVENE AND F. B. LA FORGE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

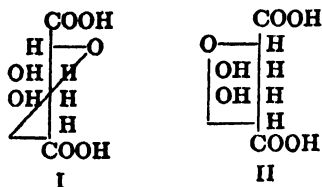
(Received for publication, April 29, 1915.)

It is known from the work of Fischer and Tiemann that on treatment of glucosamine with nitrous acid two acids are formed, depending on the mode of procedure. Chitonic acid is formed when the amino sugar is deaminized and subsequently oxidized with bromine; chitaric acid when, prior to deamination, the sugar is converted by means of bromine into glucosaminic acid. The two hexonic acids are isomeric and yield on further oxidation isosaccharic and epi-isosaccharic acids respectively. In an earlier paper Fischer¹ explained this isomerism as due to as yet undetermined differences in the configuration; in a more recent paper² he referred to the formation of the two substances as an instance of Walden's rearrangement, thus inferring that one of the acids is derived from glucosamine, the other from mannosamine. This view was based on theoretical considerations and lacked experimental support. The missing evidence has recently been furnished by the observations made on the newly discovered amino hexose, chondrosamine.³ Two isomeric acids, *l*-chondrosic (I) and *l*-epichondrosic (II), were also obtained from this sugar, depending on the order of oxidation and deamination. It was further demonstrated that these two acids correspond to two epimeric sugars: *l*-altrosamine and *l*-allosamine.

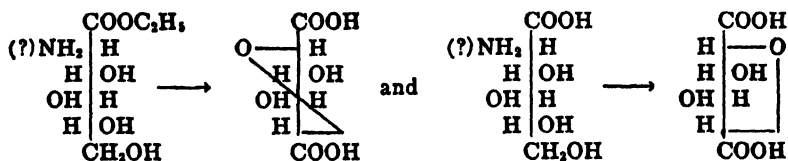
¹ E. Fischer and E. Andreae: *Ber. d. deutsch. chem. Gesellsch.*, xxxvi, p. 2587, 1903.

² E. Fischer: *Ann. d. Chem.*, ccclxxxi, p. 123, 1911.

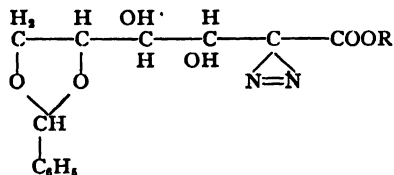
³ P. A. Levene and F. B. La Forge: *this Journal*, xx, p. 433, 1915.



Furthermore, Fischer and his coworkers⁴ have shown that α -amino-acids and their esters, on treatment with nitrous acid, do not yield the same but yield stereoisomeric hydroxy acids. Similarly the α -amino-tetrahydroxycaproic acids and their esters yield two epimeric α - α_1 -anhydro-tetrahydroxyadipic acids:



Thus convincing evidence was advanced in favor of the assumption that in either the hydroxy amino-acid or its ester, upon treatment with nitrous acid, the groups attached to the α -carbon atom undergo the Walden rearrangement. The rearrangement is brought about undoubtedly by a complex system of reactions. Fortunately, in the course of this work it was possible to isolate an intermediate substance, the diazo derivative, obtained by the action of nitrous acid upon monobenzalglucosaminic ethyl ester. On the basis of the work of Curtius⁵ the substance has the following structure:

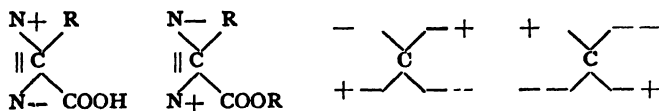


In this phase of the reaction the α -carbon atom loses its asymmetric character. In the transformation of a diazo acid into a hydroxy acid the formation of an equal mixture of the two epimeric forms is to be expected. This does not happen. Only one acid is

⁴ Fischer: *Ann. d. Chem.*, cclclxxxi, p. 123, 1911; cclclxxxvi, p. 374, 1912.

⁵ T. Curtius: *Jour. f. prakt. Chem.*, xxxix, p. 107, 1889.

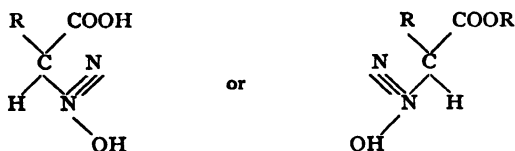
formed. Fischer,⁶ Werner,⁷ and Nef⁸ have offered theories in order to explain similar phenomena. Nef accepted a rigid difference in the four valencies of the carbon atom and expressed it in the following way:



Fischer and Werner assumed that in every reaction of substitution the place of the entering group is determined not by the position of the group being replaced, but by the nature of the remaining three groups and of their respective affinities.

The hypothesis of Nef does not provide an explanation of the phenomenon of the Walden rearrangement and those of Fischer and of Werner assume that the asymmetric carbon retains its asymmetric nature through all phases of the reaction.

It seemed to us possible that the intermediate reactions which lead to Walden's rearrangement are not identical in every instance. The hypotheses of Fischer and of Werner may be all sufficient for the understanding of the mechanism of the transformation of a halogen acid into a hydroxy acid. The transformation of an amino-acid into a hydroxy acid, or of an amino-acid into a halogen acid, on the other hand, may proceed in a different manner. For the interpretation of the reactions of the second type one may assume that the formation of the diazo derivative is followed by another intermediary phase in which one of the nitrogen atoms becomes detached from the carbon atom, while the other still remains united with it. The nitrogen atom to be detached first is determined by the nature of the other groups in combination with the asymmetric carbon atom. For instance, one may accept the intermediary formation of the following two structures:



⁶ Fischer: *Ann. d. Chem.*, ccclxxxi, p. 123, 1911; ccclxxxvi, p. 374, 1912.

⁷ A. Werner: *Ber. d. deutsch. chem. Gesellsch.*, xlv, p. 873, 1911.

⁸ J. U. Nef: *Jour. Am. Chem. Soc.*, xxx, p. 645, 1908.

This possibility is supported by the fact that aliphatic diazo acids are readily reduced to amino-acids. Attempts are now in progress to repeat the reduction experiments of Curtius on the diazo hexosaminic acids.

In principle the hypothesis advanced here does not differ from that of Fischer. Both admit the existence of the universally accepted form of static asymmetry of the carbon atom; both also admit the possibility that in certain conditions the formation of the latter form is preceded by a phase in which the asymmetry is expressed only in an uneven distribution of forces between the four valencies, and that the uneven distribution of energy is determined by the character of the groups attached to the carbon atom.

EXPERIMENTAL.

Monobenzalglucosaminic Acid Ethyl Ester Hydrochloride.

2.2 grams of finely powdered glucosaminic acid were suspended in 15 cc. of 99.7 per cent ethyl alcohol, 3 cc. of pure benzaldehyde added, and dry hydrochloric acid passed in without cooling for five minutes. After standing a short time the contents of the beaker had become filled with a mass of crystals. An equal volume of dry ether was stirred in and the product allowed to stand for a few hours at a low temperature. The crystals were then filtered off, washed with dry ether, and dried in a desiccator over potassium hydroxide. The yield was 2.65 grams. Experience showed that it was not advantageous to use more than 3 grams of glucosaminic acid for the experiment. For analysis the thoroughly dried substance was recrystallized from absolute alcohol. It crystallizes in long colorless needles which melt at 167–8° (uncorrected) with strong gas evolution.

0.1583 gm. of substance gave 5.65 cc. N, at 22°, 764 mm.

0.1108 gm. of substance gave 0.2090 gm. CO₂ and 0.0637 gm. H₂O.

	Calculated for C ₁₁ H ₁₉ O ₆ NCl:	Found:
N	4.04	4.04
C	51.80	51.50
H	6.34	6.39

Diazobenzalglucosaminic Acid Ethyl Ester.

15 grams of benzalester hydrochloride, which had been completely freed from hydrochloric acid by keeping in a desiccator over potassium hydroxide, were dissolved in 400 cc. of water, the solution was cooled to 0°, and 5 grams (about 1.5 mols.) of sodium nitrite were added. After the sodium nitrite had dissolved about 2 cc. of 50 per cent acetic acid were added. Almost immediately separation of a nearly insoluble substance began and within a few minutes the whole volume of the solution was filled with a slightly yellowish, semi-crystalline material. No appreciable gas evolution took place during the process. The product was filtered off with suction and washed with water until free from inorganic salts. When dried it appears as a light yellow powder easily soluble in alcohol, ether, and most organic solvents. It is insoluble in water; it reacts with aqueous mineral acids, and with bromine and iodine with strong gas evolution.

0.1828 gm. of substance gave 14 cc. N, at 22°, 736 mm. (Dumas).

	Calculated for $C_{12}H_{12}O_4N_2$:	Found:
N	8.69	8.66

In dry ethereal solution the product is decolorized by addition of a dry ethereal solution of hydrochloric or hydrobromic acid with vigorous evolution of nitrogen. The resulting solution probably contains as the reaction product the benzal compound of gluconic or mannonic acid ester in which the hydroxyl group on the α -carbon atom is substituted with chlorine or bromine. Upon concentration of the ethereal solution some benzaldehyde is cleaved off; complete elimination of the benzal group is accomplished by short warming on the water bath of the aqueous solution which contains a slight excess of hydrobromic acid. Benzaldehyde and benzoic acid were removed from the aqueous solution by shaking with ether, and the free bromine was removed by shaking with silver carbonate. The silver was removed from the filtrate with hydrogen sulphide and the solution concentrated in vacuum. The resulting product was a light colored syrup of strong acid properties containing fixed bromine which may be removed by heating with potassium hydroxide or silver oxide in aqueous solution. The substance has not yet been obtained in crystalline form.

XYLOHEXOSAMINIC ACID, ITS DERIVATIVES AND THEIR BEARING ON THE CONFIGURATION OF ISOSACCHARIC AND EPI-ISOSACCHARIC ACIDS.

By P. A. LEVENE AND F. B. LA FORGE.

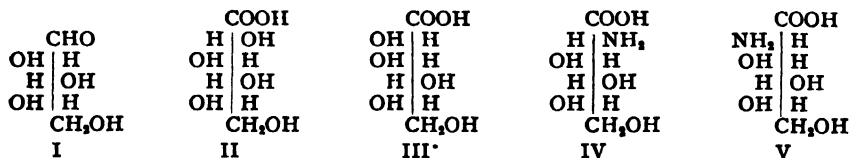
(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, April 29, 1915.)

The work on chondroitin sulphuric acid¹ is a convincing illustration of the significance of the hexosaminic acids and of the α - α_1 -anhydro-tetrahydroxyadipic acids for the progress of our knowledge of hexosamines. Every discovery of a new hexosamine will call for information on the properties and on the physical constants of the acids of these two series. The part to be played by these acids in the development of the chemistry of hexosamines is analogous to that played by the straight hexonic and by the tetrahydroxyadipic acids in that of the hexoses.

These considerations inspired the present work on the preparation of the missing hexosaminic acids and of the corresponding anhydro-tetrahydroxyadipic acids. The present communication deals with the derivatives of xylose. It was hoped through the study of its derivatives to complete our information concerning the details of the configuration of isosaccharic and of epi-isosaccharic acids.

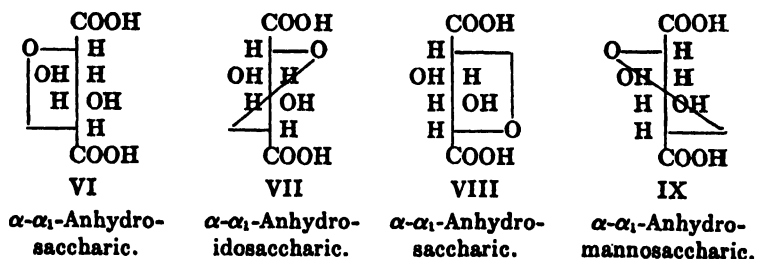
The cyanhydrin synthesis leads from xylose to two epimeric acids: idonic (II) and gulonic (III). In an analogous manner the addition of hydrocyanic acid to xylosimine should lead to two hexosaminic acids: idosaminic (IV) and gulosaminic (V).



¹ P. A. Levene and F. B. La Forge: this *Journal*, **xx**, p. 433, 1915.

In reality the formation of one of the two acids predominates to such an extent that only one can be isolated from the reaction product. It is not possible at the present state of knowledge to be certain whether the predominating substance is (IV) or (V).

When this acid is deaminized with nitrous acid and is then further oxidized by means of nitric acid it gives rise to an α - α_1 -anhydro-tetrahydroxyadipic acid. The configuration of this is easily ascertained. Gulosaminic acid, as seen from the graphic formula (V), gives rise to α - α_1 -anhydro-saccharic acid (VI), while idosaminic acid (IV) under the same conditions forms α - α_1 -anhydro-idosaccharic acid (VII). The first should be identical with the α - α_1 -anhydro-saccharic acid corresponding to glucosaminic acid (VIII). In other words, of the four α - α_1 -anhydro acids of the saccharic acid series which are obtainable from xylohexosamine and arabohehexosamine the two identical in properties correspond to glucosamine and gulosamine.



The anhydro acid obtained from xylohexosaminic acid differs in its rotation, its melting point, and in the properties of its acid potassium salt either from isosaccharic or from epi-isosaccharic acids, hence it has the configuration of α - α_1 -anhydro-idosaccharic acid (VII).

However, the knowledge of the configuration of an anhydro-tetrahydroxyadipic acid does not carry with it information on the relative position of the amino group in the parent amino-acid, so long as it is known that α -amino-acids in the process of deamination may undergo the Walden rearrangement.

The anhydro-tetrahydroxyadipic acid corresponding to the epimeric xylohexosamine was prepared principally for its bear-

ing on the configuration of isosaccharic and epi-isosaccharic acids. In a previous communication we pointed out that the present state of information did not permit a decision as to which one of the two was the anhydro-saccharic acid. The conclusion, however, would become obvious after the anhydro-tetrahydroxyadipic acid corresponding to gulosamine was obtained.

The problem was therefore reduced to the preparation of the epimer of anhydro-idosaccharic acid.

The anhydro-tetrahydroxyadipic acid obtained from glucosamine (for the present it would be more fitting to refer to it as arabohehexosamine) is epimeric with the one obtained from glucosaminic acid, and that obtained from chondrosamine is epimeric with the one obtained from chondrosaminic acid. The same method surely might have been applied successfully to xylohexosamine. However, it was thought that the substance could be obtained in a simpler manner. Fischer and his coworkers have shown that amino-acids and their esters do not yield on treatment with nitrous acid the same hydroxy acid, but yield two stereo isomers. It was, therefore, thought that the same method might be applicable to hydroxy amino-acids. In the special instance of xylohexosaminic acid the lactone was employed instead of the ester, since it offered some advantages from the standpoint of preparation. As was to be expected, in place of anhydro-idosaccharic (VII), the epimeric acid (VI) was obtained. The optical rotation, melting point, and the properties of the acid potassium salt were identical with those of epi-isosaccharic acid.

For simplicity of discussion the xylohexosaminic and the corresponding anhydro acids were presented as derived from *d*-xylose (Fischer's nomenclature; according to Rosanoff it should be termed *l*-xylose). The naturally occurring *l*-xylose leads to *l*-gulose, or to *l*-gulosamine, which form on oxidation *l*-saccharic and *l*-anhydro-saccharic acids respectively.

It may also be mentioned that the ethyl ester of xylohexosaminic acid was obtained. The substance was isolated and identified as the dibenzalxylohexosaminic ester hydrochloride. It was originally prepared for the purpose of converting it into anhydro-saccharic acid. Since this was obtained from the lactone of the hexosaminic acid, further work on the ester was abandoned. Work on lyxose derivatives is now in progress.

RÉSUMÉ.

1. From xylose, by treatment with ammonia and subsequently with hydrocyanic acid, a xylohexosaminic acid was obtained.
2. On oxidation following deamination this acid yields α - α_1 -anhydro-idosaccharic acid.
3. The lactone of the amino-acid under the same treatment leads to the α - α_1 -anhydro-saccharic acid.
4. Isosaccharic acid has the configuration of α - α_1 -anhydro-manno-saccharic acid, while epi-isosaccharic acid is the α - α_1 -anhydro-saccharic acid, and consequently,
5. Chitose and chitonic acid have the configuration of α - δ -anhydro-mannose and of α - δ -anhydro-mannonic acid respectively; chitaric acid has that of α - δ -anhydro-gluconic acid.

EXPERIMENTAL.

Xylohexosaminic Acid.

75 grams of xylosimine² were covered with 60 cc. of water, and 20 cc. of 80 per cent hydrocyanic acid were added. The mixture was warmed for about three-quarters of an hour to 35–40° and then allowed to stand at ordinary temperature for about one and a half hours, or until the contents of the flask had taken on a syrupy consistence and had darkened considerably. It was then cooled down in ice water and dissolved in 350 cc. of concentrated hydrochloric acid which had also previously been cooled to 0°, and hydrochloric acid gas passed in at room temperature until the solution was saturated. After this it was allowed to stand for about twenty hours at room temperature and finally warmed for about one and a half hours on the water bath. The solution was concentrated in vacuum to a syrup and mixed with 2 liters of absolute alcohol which caused the separation of a large part of the ammonium chloride together with a black tarry material. These were filtered off on a folded filter, and the alcoholic solution was again concentrated in vacuum to a thick syrup. (The solution was of a dark brown color at first, but became nearly colorless through the subse-

² C. A. Lobry de Bruyn and F. H. Van Leent: *Rec. d. trav. chim. d. Pays-Bas*, xiv, p. 144, 1895.

quent operations.) The syrup was dissolved in about 500 cc. of water, and after addition of sufficient hot concentrated barium hydrate solution to render it strongly alkaline, distilled in vacuum until all the ammonia was removed. At times it was necessary to dilute the residue with water and to repeat the evaporation. The final solution was then diluted to about 1 liter with water and the barium removed with a slight excess of sulphuric acid. Without filtering from barium sulphate, sulphuric and hydrochloric acids were removed with lead carbonate, hydrogen sulphide was passed through the filtrate, which was finally treated with an excess of silver carbonate, filtered, and the excess of silver removed with hydrogen sulphide. The filtrate was then concentrated in vacuum to about 50-60 cc. Out of this solution the hexosaminic acid crystallizes upon slow addition of about an equal volume of methyl alcohol. The yield of nearly pure substance was 15 grams. For analysis it was purified by solution in about 4 parts of hot water and addition of an equal volume of methyl alcohol. It crystallizes in large colorless prisms which begin to darken above 190° and decompose at about 235° (uncorrected).

0.1335 gm. of substance gave 16.8 cc. amino N, at 18°, 763 mm.

0.1214 gm. of substance gave 0.1648 gm. CO₂ and 0.0760 gm. H₂O.

	Calculated for C ₆ H ₁₂ NO ₆	Found:
C	36.92	37.01
H	6.66	7.00
N	7.18	7.26

The rotation of the substance was as follows:

$$[\alpha]_D^{30} \text{ after 20 minutes} = \frac{+0.82^\circ \times 2.1542}{1 \times 0.1500} = +11.77^\circ$$

$$[\alpha]_D^{30} \text{ after 40 hours} = \frac{-0.21^\circ \times 2.1542}{1 \times 0.1500} = -3.01^\circ$$

Lactone Hydrochloride of Xylohexosaminic Acid.

7.7 grams of amino-acid were suspended in 75 cc. of 99.5 per cent ethyl alcohol into which hydrochloric acid gas was passed, without cooling, until the alcohol was saturated. At first the substance passed into solution and immediately afterwards a small amount of a white precipitate began to form. This in turn passed into solution

as more hydrochloric acid was passed in. Finally, after the solution had become saturated, crystallization of the lactone began and was complete after the solution had stood in the cold for a few hours. The crystals were then filtered off and washed with absolute alcohol. The yield was practically quantitative, 7.7 grams giving 8 grams of lactone hydrochloride. The substance crystallizes in compact aggregates of prismatic needles grouped together in the form of spheres. When recrystallized from absolute alcohol, in which it is rather sparingly soluble, the crystals appear as long prisms. It melts at 195° (uncorrected), with decomposition and gas evolution, after it has begun to turn brown at about 180° .

0.1910 gm. of substance gave 11.5 cc. N, at 19° , 751 mm.

0.1238 gm. of substance gave 0.0829 gm. AgCl.

	Calculated for $C_{12}H_{13}NO_4Cl$:	Found:
N	6.60	6.74
Cl	16.25	16.22

Dibenzalxylohexosaminic Acid Ester Hydrochloride.

0.75 gram of amino-acid was suspended in 15 cc. of absolute alcohol and hydrochloric acid gas was passed into the suspension, without cooling, until complete solution had taken place. At this stage 2 cc. of pure benzaldehyde were added and more hydrochloric acid gas was passed into the solution. Very soon the contents of the flask were filled with a mass of crystals. These were filtered off, washed with absolute alcohol, and dried in a desiccator over potassium hydroxide. The yield was 1.2 grams. The completely dried product was recrystallized from a large amount of absolute alcohol. It melted at 217° (uncorrected). The analysis indicates that two benzaldehyde groups have entered into the reaction.

0.2116 gm. of substance gave 6.0 cc. N, at 19° , 760 mm.

0.1297 gm. of substance gave 0.2875 gm. CO_2 and 0.0690 gm. H_2O .

	Calculated for $C_{22}H_{24}NO_6Cl$:	Found:
C	60.65	60.47
H	6.34	6.39
N	3.20	3.25

α - α_1 -Anhydro-idosaccharic Acid.

9 grams of xylohexosaminic acid were dissolved in 100 cc. of 3 per cent hydrochloric acid and deaminized with 9 grams of silver nitrite. After standing for about twenty-four hours the slight excess of silver was removed with a little hydrochloric acid, the silver chloride filtered off, and the filtrate concentrated in vacuum to about 20 cc. An equal volume of concentrated nitric acid was added and the solution boiled over a small flame for twelve minutes. It was then transferred to a shallow dish and rapidly concentrated on the water bath to a thick syrup. This was taken up in a small amount of water and again evaporated to remove the excess of nitric acid. Crystallization took place spontaneously during the second evaporation. After standing for a short time the crystals were washed from adhering syrup with a mixture of 1 part of dry acetone + 3 parts of dry ether, filtered, and dried in a desiccator. The yield was 3.5 grams. It was recrystallized for analysis by dissolving in 10 parts of dry acetone and adding an equal volume of dry ether. The substance crystallizes under these conditions in long colorless prismatic needles, which melt at 226° (uncorrected), with gas evolution. The product contains 2 molecules of crystal water which may be removed by heating in vacuum at 108° .

- I. 0.1220 gm. of substance gave 0.0188 gm. H_2O .
 II. 0.1240 gm. of substance gave 0.0192 gm. H_2O .
 III. 0.1018 gm. of substance gave 0.0160 gm. H_2O .

	Calculated for $C_6H_8O_7 + 2H_2O$:	I.	Found: II.	III.
H_2O	15.80	15.53	15.48	15.73

0.1032 gm. of dry substance gave 0.1432 gm. CO_2 and 0.0410 gm. H_2O .

	Calculated for $C_6H_8O_7$:	Found:
C	37.50	37.79
H	4.20	4.45

0.1040 gm. of crystal water containing substance required 9.0 cc. $\frac{N}{10}$ NaOH (calculated 9.1 cc.).

The rotation of the dry substance was as follows:

$$[\alpha]_D^{20} = \frac{-7.6^\circ \times 2.1728}{1.033 \times 1 \times 0.1713} = -93.32^\circ$$

The acid forms an acid potassium salt which is obtained by neutralizing the acid in concentrated aqueous solution with potassium hydrate, adding an equal volume of glacial acetic acid and suffi-

cient alcohol to produce a permanent turbidity. The salt crystallizes in long colorless needles, which, owing to their great solubility in water, are difficult to free from sodium acetate. For this reason no analysis of the product is given.

0.1414 gm. of substance in 2 cc. of water rotated -6.14° .

α - α_1 -l-Anhydro-Saccharic Acid (l-Epi-isosaccharic Acid³).

3.5 grams of the above mentioned hydrochloride of xylohexosaminic acid lactone were dissolved in 50 cc. of water and deaminized with 4 grams of silver nitrite. The reaction mixture was kept at 0° for the first five hours and then allowed to stand over night at room temperature. If silver was present in the solution it was removed with a few drops of hydrochloric acid, the silver chloride filtered off, and the filtrate concentrated in vacuum to about 15 cc. An equal volume of concentrated nitric acid was then added and the solution boiled over a small flame for about twelve minutes. It was then evaporated in a flat dish on the water bath to a syrup which was freed from most of the nitric acid by repeating the evaporation after adding a small amount of water. The reaction product was taken up in about 6 cc. of water, neutralized with a strong solution of potassium hydroxide, and allowed to stand fifteen minutes at room temperature, after which an equal volume of glacial acetic acid and about 3 volumes of absolute alcohol were added. Upon standing in the refrigerator from four to five hours crystallization of the acid potassium salt was complete. The yield was 1.6 grams. It was recrystallized from 2 parts of hot water.

0.2314 gm. of substance gave 0.0797 gm. K_2SO_4 .

	Calculated for $C_6H_7O_7K + H_2O$:	Found:
K	15.70	15.45

The rotation of the air-dried substance⁴ was as follows:

$$[\alpha]_D^{28} = \frac{-2.54^{\circ} \times 2.1114}{1 \times 0.1400} = -38.09^{\circ}$$

³ In giving the rotation of the antipode (this *Journal*, xx, p. 444, 1915), we omitted to mention the weight of the solution used, which was 2.1478 grams.

⁴ For comparison we determined the rotation of the same salt of *d*-anhydro-saccharic acid obtained from glucosaminic acid (this *Journal*, xx, p. 442, 1915).

$$[\alpha]_D^{28} = \frac{+2.56^{\circ} \times 2.1221}{1 \times 0.1410} = +38.53^{\circ}$$

4 grams of the acid potassium salt were dissolved in about 150 cc. of water and the calculated amount of a 5 per cent solution of lead acetate was added. Crystallization of the lead salt began at once and was complete after about one hour. The lead salt was filtered off with suction, washed with water, and dried at 100°. The yield was 6 grams. The lead salt was suspended in about 100 cc. of water and slightly less than the calculated amount of sulphuric acid added. After standing about one hour on the water bath the lead sulphate was filtered off and the filtrate concentrated in vacuum to a thick syrup which was dissolved in dry acetone and again evaporated to dryness. The syrupy residue, which contained lead salts, was extracted with dry acetone, and the colorless filtrate evaporated in a dish on the water bath to a thick syrup. By repeating the evaporation with acetone a few times the syrup crystallized on cooling without crystal water. It was freed from a small amount of adhering syrup by washing with a mixture of 1 part of amyl alcohol + 2 parts of dry ether. The yield was 1.5 grams. It may be recrystallized by dissolving in a small amount of dry acetone and allowing the solution to evaporate in the air. The substance melts at 163°.

0.1092 gm. of substance gave 0.1514 gm. CO₂ and 0.0410 gm. H₂O.

	Calculated for C ₆ H ₈ O ₇ :	Found:
C	37.50	37.80
H	4.20	4.20

The rotation of the substance was as follows:

$$[\alpha]_D^{21} = \frac{-3.08^\circ \times 2.1547}{1.034 \times 0.1657} = -38.79^\circ$$

THE QUATERNARY SALTS OF HEXAMETHYLENE- TETRAMINE.

V. MONOHALOGENACETYL DERIVATIVES OF AMINOALCOHOLS AND THE HEXAMETHYLENETETRAMINIUM SALTS DERIVED THEREFROM.¹

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The only reference to be found in the literature to the halogen-acetyl derivatives of the aminoalcohols or alcamines is that contained in the work of Einhorn and his collaborators.² In the communications of these authors the halogenacetyl derivatives of the simplest alcamine, aminomethanol, are described, and along with these the quaternary salts obtained by combining them with hexamethylenetetramine. The present authors have had occasion to work somewhat extensively in this field and have made the results of these investigations the subject of the present communication.

In the biological studies with which the chemical work was associated, the results obtained at the time with certain members of this class of hexamethylenetetramine quaternary salts were such as to demand a detailed study of the field. It had been hoped at first that by concentrating our attention on the simplest member of the series, chloroacetylaminomethanol, in the way of acylating the hydroxyl group with a great variety of acids, a uniform series of substances would be obtained for biological study.

The acylation of the hydroxyl group to form chloroacetylamino-

¹ For the preceding papers of this series, see this *Journal*, xx, pp. 659 and 685; xxi, pp. 103 and 145.

² A. Einhorn and collaborators: *Ann. d. Chem.*, cccxliii, p. 207, 1905; cccxli, p. 113, 1908.

methyl esters was readily accomplished by the use of the pyridine method. In attempting, however, to combine these esters with hexamethylenetetramine, although addition occurred, we found it impossible to isolate the product sought. In each case the product obtained was the quaternary salt of the base with chloroacetylaminomethanol. It is possible that moisture was responsible for the saponification of the quaternary ester salt which was first formed.

By turning to the higher aminoalcohols the results obtained were more promising. Both the chloroacetyl aminoalcohols and the esters derived from them reacted smoothly with hexamethylenetetramine to form stable salts. With chloroacetyl aminoethanol, in particular, a large number of esters were prepared and combined with hexamethylenetetramine, forming a uniform series of compounds suited for the biological work. The chloroacetyl aminoalcohols were prepared from the aminoalcohols and chloroacetyl chloride by the Schotten-Baumann method, and, in the case of the aliphatic compounds, were purified by distillation in high vacuum. The esters of chloroacetyl aminoethanol were prepared by the acylation of this substance with the acid chloride in pyridine solution, and in some cases, though less advantageously, by the chloroacetylation of the aminoethyl esters obtained by Gabriel's method.³

This last procedure was also attempted with the esters of γ -aminopropanol and aminoisopropylalcohol, but the chloroacetylation by the Schotten-Baumann method was found to be uncertain. The aminopropyl esters obtained by the rearrangement of the bromopropylamides according to Gabriel's method were isolated as the hydrobromides. In attempts to chloroacetylate these with chloroacetyl chloride and alkali, the aminopropyl ester liberated by the alkali as free base rapidly rearranged to the oxypropylamide, and the product obtained was either a mixture of the chloroacetylaminopropyl ester and the chloroacetate of oxypropylamide or the latter alone. In the case of the aminoisopropyl ester of *p*-nitrobenzoic acid the rapidity of this rearrangement was very striking. The most suitable method for obtaining the chloroacetylaminopropyl esters, provided the parent aminoalcohols are accessible, is by direct acylation of the chloroacetyl aminoalcohols..

³ S. Gabriel and P. Heymann: *Ber. d. deutsch. chem. Gesellsch.*, xxiii, p. 2493, 1890.

The work was extended to include the derivatives of secondary aminoalcohols with the nitrogen substituted both by alkyl and aryl groups. The chloroacetyl derivatives of this type either did not react with hexamethylenetetramine or reacted so slowly that only in one or two cases was it possible to isolate a crystalline salt. Although the bromo- and iodo-derivatives reacted more rapidly, we were unable to isolate the products formed in crystalline condition. The bromo- and iodo-derivatives themselves proved to be substances difficult to obtain crystalline.

EXPERIMENTAL.

As in the preceding papers of this series, the hexamethylenetetraminium salts were prepared, unless otherwise stated, by boiling equimolecular amounts of the components in a small volume of dry, alcohol-free chloroform. With one or two exceptions, which are noted as they occur, the hexamethylenetetraminium salts described in this communication are readily soluble in water. We wish also to reiterate here what was stated in the first paper, that the melting points or rather decomposition points of these quaternary salts depend greatly upon the mode of heating and that for this reason the determination of the corrected melting points was useless.

Unless otherwise stated, the following method was used for the preparation of the esters of the chloroacetyl aminoalcohols described below. This is given here in order to avoid unnecessary repetition. The chloroacetyl aminoalcohol was dissolved in five parts by weight of pyridine, cooled in a freezing mixture, and carefully treated with a solution of 1.25 mols. of the acid chloride in a little dry, alcohol-free ether or chloroform. After standing for fifteen to thirty minutes at room temperature, the mixture was poured into iced 25 per cent sulphuric acid. If the reaction mixture is allowed to stand too long the yields are reduced, apparently owing to the formation of soluble pyridinium salts. The esters generally separated as oils which rapidly solidified, in some cases only after the removal of the ether or chloroform by means of a current of air. The crude products were thoroughly washed first with dilute sulphuric acid, then with 5 per cent sodium carbonate solution, and finally with water.

Variations from the above procedure are denoted below as they occur.

Halogen in the intermediate products was determined by the Volhard method after hydrolysis with 10 per cent alcoholic sodium hydroxide.

(1) *Oxymethylchloroacetamide and Derivatives.*

Oxymethylchloroacetamide and hexamethylenetetramine. This substance, already obtained by Einhorn and Göttler,⁴ was more conveniently prepared by us as follows: Equimolecular amounts of the components were boiled for one and one-half to two hours in about ten parts of acetone. The precipitated salt was filtered off and washed well with dry chloroform. An additional amount was obtained by continued boiling of the filtrate from the first crop. As prepared in this way, the salt melts with decomposition at 154–5° and is not hygroscopic, as stated by the above authors. Yield: almost quantitative.

0.2435 gm. of substance required 9.13 AgNO₃ Solution I.⁵

Calculated for C₆H₁₀O₂N₂Cl: Cl = 13.45 per cent.

Found: Cl = 13.20 per cent.

Chloroacetylaminomethyl Benzoate, ClCH₂CONHCH₂OOC C₆H₅.

—The ester, obtained by the method outlined above, was recrystallized twice from absolute alcohol, then once from toluene. It forms prismatic rods which melt at 73–3.5° (corrected) with slight preliminary softening. It is very soluble in chloroform, less easily in ether and benzene.

0.2335 gm. of substance (Kjeldahl) required 10.2 cc. $\frac{N}{10}$ HCl.

0.1468 gm. of substance required 12.09 cc. AgNO₃ Solution II.⁶

Calculated for C₁₀H₁₀O₂NCl: N = 6.16 per cent; Cl = 15.58 per cent.

Found: N = 6.12 per cent; Cl = 15.32 per cent.

Attempts to prepare the hexamethylenetetraminium salt of the compound by the usual methods resulted only in the isolation of the impure quaternary salt of oxymethylchloroacetamide.

⁴ A. Einhorn and M. Göttler: *Ann. d. Chem.*, ccclxi, p. 150, 1908.

⁵ 1 cc. = 0.00352 gm. Cl; 0.00793 gm. Br; 0.01259 gm. I.

⁶ 1 cc. = 0.00186 gm. Cl; 0.004192 gm. Br; 0.006657 gm. I.

Chloroacetylaminomethyl Anisate (*Chloroacetylaminomethyl p-methoxybenzoate*).—The crude ester, obtained in practically quantitative yield, was recrystallized first from absolute alcohol, then benzene containing a little toluene to prevent its freezing in the ice box. It forms large rosettes of delicate hairs which melt at $106-7^{\circ}$ (corrected) with slight preliminary sintering. It is somewhat soluble in ether, very easily in chloroform.

0.2089 gm. of substance (Kjeldahl) required 8.25 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{11}H_{12}O_4NCl$: N = 5.44 per cent.

Found: N = 5.53 per cent.

Attempts to prepare the quaternary salt by the usual methods failed as in the case of the benzoate.

(2) *Derivatives of Chloroacetylamin ethanol.*

Chloroacetylamin ethanol, $ClCH_2CONHCH_2CH_2OH$.—25 grams of redistilled aminoethyl alcohol (Kahlbaum) were dissolved in 100 cc. of water and treated with 225 cc. of two-normal sodium hydroxide solution. After cooling in a freezing mixture, 50 grams of chloroacetyl chloride were added drop by drop, with vigorous shaking and continued cooling. At the end, an excess of 10 per cent sodium hydroxide solution was added until the reaction was definitely alkaline, after which the mixture was neutralized with hydrochloric acid and concentrated as far as possible *in vacuo*. The residue was taken up in dry acetone and filtered from the sodium chloride. The filtrate was again concentrated and the treatment with dry acetone repeated. After again concentrating the filtrate the residue was fractionated *in vacuo*. The chloroacetylamin ethanol boils at $141-5^{\circ}$ (corrected, bath at $180-90^{\circ}$) at 0.25–0.35 mm. and solidifies in a freezing mixture, melting again unsharply as the temperature is raised. Yield: 75 per cent of the theory. The compound is readily soluble in water, alcohol, and acetone, difficultly in chloroform.

0.2550 gm. of substance (Kjeldahl) required 18.01 cc. $\frac{N}{10}$ HCl.

0.1452 gm. of substance required 19.98 cc. $AgNO_3$ Solution II.

Calculated for $C_4H_8O_2NCl$: N = 10.18 per cent; Cl = 25.78 per cent.

Found: N = 9.90 per cent; Cl = 25.61 per cent.

Iodoacetylamin ethanol.—1 mol. of chloroacetylamin ethanol was dissolved in a solution of 1.1 mols. of sodium iodide in dry acetone and allowed to stand for twenty-four hours. The precipitated sodium chloride was filtered off and the filtrate concentrated to a syrup. Although all attempts at further purification were unsuccessful, the crude product was sufficiently pure for the preparation of the two following salts.

Iodoacetylamin ethanol and Hexamethylenetetramine.—The crude iodo-compound was dissolved in absolute alcohol and added to a cold solution of the base in the minimum amount of absolute alcohol. The salt, which soon began to separate, was filtered off after twenty-four hours and washed with absolute alcohol. It melts with decomposition at $142-3^{\circ}$.

0.1968 gm. of substance required 5.43 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{10}\text{H}_{20}\text{O}_2\text{N}_2\text{I}$: I = 34.35 per cent.

Found: I = 34.73 per cent.

Iodoacetylamin ethanol and Trimethylamine.—For purposes of comparison this salt was prepared in the same way as the hexamethylenetetraminium compound. It sinters at 135° and melts at $138-9^{\circ}$ (corrected). It dissolves readily in water and warm alcohol.

0.1954 gm. of substance required 6.85 cc. AgNO_3 Solution I.

Calculated for $\text{C}_7\text{H}_{17}\text{O}_2\text{N}_3\text{I}$: I = 44.05 per cent.

Found: I = 44.14 per cent.

Chloroacetylamin ethyl Benzoate.—In addition to the method given above for the preparation of esters of chloroacetylamin ethanol, the Schotten-Baumann method, starting with aminoethyl benzoate hydrobromide and chloroacetyl chloride, may be employed. The yield resulting from this procedure is poor, however, owing to the rapid rearrangement of the free base to the oxyethylbenzamide. Recrystallized first from absolute alcohol, then toluene, the ester forms flat needles which soften at 68.5° and melt at $69-9.5^{\circ}$ (corrected). It is very soluble in chloroform, less so in benzene and ether.

0.2135 gm. of substance (Kjeldahl) required 8.8 cc. $\frac{N}{10}$ HCl.

Calculated for $\text{C}_{11}\text{H}_{15}\text{O}_2\text{NCl}$: N = 5.80 per cent.

Found: N = 5.77 per cent.

Chloroacetylaminioethyl Benzoate and Hexamethylenetetramine.—Equimolecular proportions of the components were heated in a small amount of dry chloroform for one hour. The addition of dry acetone resulted in the precipitation of the salt as a paste. This became crystalline on warming and rubbing. The compound darkens at about 158° and melts with decomposition at 162° .

0.2006 gm. of substance required 5.5 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{11}\text{H}_{14}\text{O}_3\text{N}_4\text{Cl}$: Cl = 9.29 per cent.

Found: Cl = 9.65 per cent.

Chloroacetylaminioethyl o-Toluate.—Recrystallized successively from 50 per cent alcohol, absolute alcohol, and toluene, the ester forms faintly pinkish clusters of spears which soften at 64.5° and melt at $65-5.5^{\circ}$ (corrected), the melting point being unchanged by a subsequent recrystallization from absolute alcohol. It is extremely soluble in chloroform, less so in ether and benzene.

0.2065 gm. of substance (Kjeldahl) required 8.25 cc. $\frac{N}{16}$ HCl.

Calculated for $\text{C}_{12}\text{H}_{14}\text{O}_3\text{NCl}$: N = 5.48 per cent.

Found: N = 5.60 per cent.

Chloroacetylaminioethyl o-Toluate and Hexamethylenetetramine.—After one hour's boiling in dry chloroform solution the salt separated on cooling. It melts at 168° with decomposition.

0.2096 gm. of substance required 5.38 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{18}\text{H}_{20}\text{O}_3\text{N}_4\text{Cl}$: Cl = 8.96 per cent.

Found: Cl = 9.03 per cent.

Chloroacetylaminioethyl p-Toluate.—Recrystallized first from absolute alcohol, then toluene, the ester forms glistening, felted hairs which are rather less soluble in the usual organic solvents than is the *ortho* isomer. It melts at $106-7.5^{\circ}$ (corrected).

0.2081 gm. of substance (Kjeldahl) required 8.1 cc. $\frac{N}{16}$ HCl.

Calculated for $\text{C}_{12}\text{H}_{14}\text{O}_3\text{NCl}$: N = 5.48 per cent.

Found: N = 5.45 per cent.

Chloroacetylaminioethyl p-Toluate and Hexamethylenetetramine.—After one hour's boiling, the salt was precipitated from the chloroform solution by means of dry acetone. When rapidly heated it

melts to an orange liquid at $161-2^{\circ}$, but melts at a much lower temperature when slowly heated.

0.2030 gm. of substance required 5.22 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{18}\text{H}_{20}\text{O}_2\text{N}_2\text{Cl}$: $\text{Cl} = 8.96$ per cent.

Found: $\text{Cl} = 9.05$ per cent.

Chloroacetylaminooethyl β -Naphthoate.— β -Naphthoyl chloride was obtained in excellent yield from the acid and thionyl chloride. The ester prepared with its aid, when recrystallized first from absolute alcohol, then twice from benzene, forms minute, voluminous, strongly triboelectric needles which melt at $137-9^{\circ}$ (corrected). This method of purification removes an impurity crystallizing in the form of plates, which was not further investigated. The ester is sparingly soluble in cold acetone, readily in acetic acid.

0.1877 gm. of substance (Kjeldahl) required 6.7 cc. $\frac{N}{10}$ HCl .

Calculated for $\text{C}_{18}\text{H}_{18}\text{O}_2\text{NCl}$: $\text{N} = 4.80$ per cent.

Found: $\text{N} = 5.00$ per cent.

Chloroacetylaminooethyl β -Naphthoate and Hexamethylenetetramine.—After concentration of the chloroform solution to small bulk, precipitation of the salt was completed by the addition of several volumes of dry acetone. The compound was purified by boiling for one hour with dry acetone. It melts and decomposes at $174-6^{\circ}$. When once isolated it is difficultly soluble in chloroform.

0.2092 gm. of substance required 4.84 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{21}\text{H}_{20}\text{O}_2\text{N}_2\text{Cl}$: $\text{Cl} = 8.22$ per cent.

Found: $\text{Cl} = 8.14$ per cent.

Chloroacetylaminooethyl o-Nitrobenzoate.—*o*-Nitrobenzoyl chloride prepared from the acid and thionyl chloride was used. As the crude ester could not be made to crystallize, it was taken up with ether, washed with small amounts of 10 per cent sulphuric acid until all pyridine had been removed, then with 5 per cent sodium carbonate solution, and finally with water. After drying over sodium sulphate the solvent was removed, finally *in vacuo*, but the resulting syrup could not be obtained crystalline. It is miscible in all proportions with chloroform and is insoluble in ligroin.

0.2034 gm. of substance (Kjeldahl) required 13.60 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{11}H_{11}O_2N_2Cl$: N = 9.78 per cent.

Found: N = 9.37 per cent.

Chloroacetylaminioethyl o-Nitrobenzoate and Hexamethylenetetramine.—The solution of the components in dry, boiling chloroform soon set to a solid cake. This was disintegrated, after which the heating was continued for one-half hour. Additional quantities of the salt were obtained from the mother liquors on continued boiling. As precipitated, it contains chloroform of crystallization, one molecule of which is retained even on heating *in vacuo* at the temperature of boiling alcohol over sulphuric acid. A strong odor of chloroform is apparent when the salt is dissolved in water.

0.3590 gm. of substance required 6.89 cc. AgNO₃ Solution I.

Calculated for $C_{17}H_{22}O_5N_6Cl \cdot CHCl_3$: Cl = 6.49 per cent.

Found: Cl = 6.75 per cent.

The chloroform of crystallization may be removed by boiling the salt for one hour in dry acetone. It then melts with decomposition at 174° when plunged into the bath at 165°.

0.2094 gm. of substance required 4.98 cc. AgNO₃ Solution I.

Calculated for $C_{17}H_{22}O_5N_6Cl$: Cl = 8.31 per cent.

Found: Cl = 8.37 per cent.

Chloroacetylaminioethyl m-Nitrobenzoate.—The ester could not be obtained crystalline. Even when purified as in the case of the *ortho* isomer it was still very impure, as shown by analysis.

Chloroacetylaminioethyl m-Nitrobenzoate and Hexamethylenetetramine.—The chloroform solution was boiled for about two and a half hours, filtered from a small amount of what was probably hexamethylenetetraminium chloride, and treated with dry acetone. The salt separated as an oil which soon crystallized. The product was filtered off and warmed several hours with dry acetone.

0.2273 gm. of substance required 5.96 cc. AgNO₃ Solution I.

Calculated for $C_{17}H_{22}O_5N_6Cl$: Cl = 8.31 per cent.

Found: Cl = 9.23 per cent.

Chloroacetylaminioethyl p-Nitrobenzoate.—Recrystallized from absolute alcohol, the ester forms long, prismatic needles which melt at

127–8° (corrected). It is difficultly soluble in cold absolute alcohol and benzene, more easily in acetone and chloroform.

0.1969 gm. of substance required 6.8 cc. AgNO₃ Solution I.

Calculated for C₁₁H₁₁O₅N₂Cl: Cl = 12.38 per cent.

Found: Cl = 12.16 per cent.

Chloroacetylaminioethyl p-Nitrobenzoate and Hexamethylenetetramine.—The salt separates from the chloroform solution. It melts at 182–2.5° with decomposition.

0.1985 gm. of substance required 4.55 cc. AgNO₃ Solution I.

Calculated for C₁₇H₂₃O₅N₆Cl: Cl = 8.31 per cent.

Found: Cl = 8.07 per cent.

Chloroacetylaminioethyl p-Nitrobenzoate and Trimethylamine.—The ester and an excess of trimethylamine were heated in dry chloroform at 100° for one hour in a sealed tube. The resulting clear solution was evaporated to small bulk and treated first with a little dry acetone, then with dry ether. The salt separated as a white, crystalline powder melting at 175–6° (corrected). When once isolated it is insoluble in chloroform and acetone, but dissolves easily in water and in alcohol.

0.1473 gm. of substance required 4.3 cc. AgNO₃ Solution I.

Calculated for C₁₄H₂₀O₅N₃Cl: Cl = 10.26 per cent.

Found: Cl = 10.28 per cent.

Chloroacetylaminioethyl p-Aminobenzoate.—12 grams of chloroacetylaminioethyl *p*-nitrobenzoate were added slowly to a solution of 50 grams of stannous chloride in 100 cc. of acetic acid saturated with hydrochloric acid gas. The temperature rose rapidly, but was kept below 30° with the aid of a freezing mixture. The reaction mixture, from which a double salt had partially separated as a gummy mass, was poured into ice water and the solution treated with 50 per cent sodium hydroxide solution until the tin precipitate redissolved, taking care that the solution remained ice cold in order to prevent saponification of the ester. In a second experiment the ester crystallized out at this point in the form of glistening platelets which were immediately filtered off, but in the first trial it was necessary to shake out the alkaline solution with chloroform in order to isolate

the compound. After drying the chloroform solution over sodium sulphate it was concentrated to a syrup. When this was taken up in a little hot alcohol and cooled, it set to a solid cake. This was broken up with a little dry ether and filtered. Yield: 7 grams. The ester was recrystallized twice from absolute alcohol, using bone-black the first time. It forms pale cream-colored, minute spindles which melt at $117-8.5^{\circ}$ (corrected) with preliminary softening. It is very difficultly soluble in ether, benzene, toluene, and cold acetone.

0.1514 gm. of substance (Kjeldahl) required 11.55 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{11}H_{13}O_3N_2Cl$: N = 10.92 per cent.

Found: N = 10.69 per cent.

Chloroacetylaminoethyl p-[Azodiethylaniline]-benzoate (*Chloroacetylaminoethyl Ester of p*-Carboxybenzeneazo-*p*'-diethylaminobenzene).—



4 grams of chloroacetylaminoethyl *p*-aminobenzoate were dissolved at 0° in 35 cc. of normal hydrochloric acid. With continued cooling the solution was diazotized with a solution of 1.1 grams of sodium nitrite in a little water. After stirring for ten minutes, sodium acetate was added until the reaction was neutral to Congo Red paper, after which a solution of 4.8 grams of diethylaniline in 50 cc. of alcohol was added. Coupling commenced at once, with separation of a tar which partially crystallized on scratching, forming microscopic rosettes of platelets. The addition of one-half volume of water completed the separation of the dye and resulted in the crystallization of the entire product. This was filtered off, washed with 50 per cent alcohol, and dissolved in a little hot absolute alcohol. The substance separated as an oil on cooling, but was converted into aggregates of red, microscopic crystals by the cautious addition of water, accompanied by rubbing. After standing in the ice box over night the dye was filtered off, washed with 50 per cent alcohol, and dried. The yield was practically quantitative. The dye sinters at 111° and gradually melts until it is completely fluid at 116° .

0.1691 gm. of substance (Kjeldahl) required 16.36 cc. $\frac{N}{N}$ HCl.

Calculated for $C_{21}H_{28}O_8N_4Cl$: N = 13.45 per cent.

Found: N = 13.56 per cent.

Chloroacetylaminioethyl p-[Azodiethylaniline]-benzoate and Hexamethylenetetramine.—Equimolecular amounts of the components were boiled in a little dry chloroform for one-half hour, after which the mixture set to a thick paste. By the cautious addition of dry acetone this was converted into a light orange, crystalline mass. As first obtained, this seemed to be entirely soluble in water, but when dried the salt was only incompletely soluble. It melts at 179–81°. The aqueous solution dyes silk a bright orange shade.

0.3163 gm. of substance gave 0.0828 gm. AgCl.

Calculated for $C_{27}H_{37}O_8N_4Cl$: Cl = 6.37 per cent.

Found: Cl = 6.47 per cent.

Chloroacetylaminioethyl Acetylsalicylate.—The acetylsalicylic chloride used for this preparation was prepared according to Anschütz.⁷ The crude ester was dried *in vacuo* and recrystallized from benzene, forming glistening, snow-white tablets, melting at 117° (corrected) with slight preliminary softening. It is soluble in the cold in absolute alcohol, difficultly in ethyl acetate.

0.2033 gm. of substance (Kjeldahl) required 7.0 cc. $\frac{N}{N}$ HCl.

Calculated for $C_{18}H_{18}O_6N_2Cl$: N = 4.73 per cent.

Found: N = 4.82 per cent.

Chloroacetylaminioethyl Acetylsalicylate and Hexamethylenetetramine.—The clear chloroform solution obtained after boiling for one and a half hours was evaporated to small bulk. Several volumes of dry acetone were then added. The salt remained in solution until this was seeded with crystals obtained in a test portion by the addition of dry ether. The product forms micro-platelets which melt to a yellowish liquid at 154–6° with slight gas evolution. It dissolves readily in chloroform, alcohol, and water.

0.2180 gm. of substance required 5.14 AgNO₃ Solution I.

Calculated for $C_{19}H_{18}O_6N_2Cl$: Cl = 8.07 per cent.

Found: Cl = 8.30 per cent.

⁷ R. Anschütz: *Ann. d. Chem.*, ccclxvii, p. 173, 1909.

Chloroacetylaminioethyl Anisate (*Chloroacetylaminioethyl p-Methoxybenzoate*).—In addition to the usual method of preparation, this ester may be obtained, although in poor yield, from aminoethyl anisate hydrobromide and chloroacetyl chloride by the Schotten-Baumann method. Recrystallized first from dilute alcohol, then benzene containing a little toluene to prevent its freezing in the ice box, and finally from absolute alcohol, the ester melts constantly at $96.5-7^{\circ}$ (corrected) with slight preliminary softening. It forms rosettes of needles which are rather difficultly soluble in cold ether, benzene, and alcohol, but dissolve readily in chloroform.

0.1830 gm. of substance (Kjeldahl) required 6.85 cc. $\frac{N}{10}$ HCl.

0.1951 gm. of substance required 13.58 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{12}\text{H}_{14}\text{O}_4\text{NCl}$: N = 5.16 per cent; Cl = 13.05 per cent.

Found: N = 5.24 per cent; Cl = 12.95 per cent.

Chloroacetylaminioethyl Anisate and Hexamethylenetetramine.—After one hour's boiling the salt was precipitated from the chloroform solution by means of dry acetone. It melts at $153-4^{\circ}$ with decomposition and gives a pale pink color in sulphuric acid.

0.1528 gm. of substance required 3.7 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{18}\text{H}_{26}\text{O}_4\text{N}_6\text{Cl}$: Cl = 8.61 per cent.

Found: Cl = 8.52 per cent.

Chloroacetylaminioethyl Cinnamate.—Recrystallized first from absolute alcohol, then toluene, then absolute alcohol, the ester melts constantly at $120-2^{\circ}$ (corrected). It forms delicate, glistening needles which are difficultly soluble in the cold in ether, alcohol, benzene, and toluene.

0.2677 gm. of substance (Kjeldahl) required 10.08 cc. $\frac{N}{10}$ HCl.

0.1456 gm. of substance required 10.05 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{18}\text{H}_{18}\text{O}_3\text{NCl}$: N = 5.24 per cent; Cl = 13.25 per cent.

Found: N = 5.26 per cent; Cl = 12.88 per cent.

Chloroacetylaminioethyl Ethyl Ether,



25 cc. of two-normal sodium hydroxide solution were added to a cooled solution of 3 grams of freshly distilled aminoethyl ethyl ether^a

^a L. Knorr: *Ber. d. deutsch. chem. Gesellschaft.*, xxxvii, p. 3506, 1904.

in a little water. The mixture was chilled with salt and ice and chloroacetylated with 2 mols. of chloroacetyl chloride, adding alkali from time to time as necessary. At the end, solid potassium carbonate was added until the chloroacetyl derivative separated as an oil. This was taken up in chloroform and the aqueous layer extracted five times with the same solvent. The chloroform solution was dried over sodium sulphate and evaporated to small bulk. Almost all of the residue boiled at 132° (corrected) at 15 mm. Yield: 3.5 grams.

0.1266 gm. of substance (Kjeldahl) required 7.55 cc. $\frac{N}{10}$ HCl.

0.0957 gm. of substance required 11.13 cc. AgNO₃ Solution II.

Calculated for C₆H₁₁O₂NCl: N = 8.46 per cent; Cl = 21.41 per cent.

Found: N = 8.35 per cent; Cl = 21.64 per cent.

Chloroacetylaminioethyl Ethyl Ether and Hexamethylenetetramine.

—The salt was precipitated from the chloroform solution by the addition of dry acetone and rubbing. It was filtered off, washed with dry acetone, and rapidly transferred to a desiccator as it proved to be very hygroscopic. It melted unsharply at $135-45^{\circ}$.

0.1979 gm. of substance required 12.2 cc. AgNO₃ Solution II.

Calculated for C₁₂H₂₄O₂N₃Cl: Cl = 11.60 per cent.

Found: Cl = 11.47 per cent.

Aminoethyl o-Tolyl Ether (o-Methylphenoxyethylamine),



60 grams of *o*-methylphenoxyethyl bromide were heated at 120° for four hours in an autoclave with 600 cc. of alcoholic ammonia saturated at 0° . The ammonia and alcohol were boiled off, finally with steam, leaving a solution containing oil droplets. These (probably the imino compound) were removed by shaking out with ether. The aqueous layer was then made alkaline and shaken out again with ether. The residue obtained after evaporating off the ether was fractionated *in vacuo*. The amine boils at 128° (corrected) at 19 mm. and has an unpleasant odor. It is almost insoluble in cold water, but dissolves more freely on heating. It gives a pale pinkish color in sulphuric acid.

0.2542 gm. of substance (Kjeldahl) required 16.85 cc. $\frac{N}{10}$ HCl.

Calculated for $C_9H_{11}ON$: N = 9.27 per cent.

Found: N = 9.28 per cent.

Chloroacetylaminioethyl o-Tolyl Ether.—To a solution of 10 grams of the amine in benzene were added an excess of two-normal, aqueous sodium hydroxide and, with cooling and vigorous shaking, a solution of 10 grams of chloroacetyl chloride in dry benzene. The mixture was then acidified and the benzene layer washed with water and sodium carbonate solution. After drying it was evaporated to small bulk. The resulting syrup (14 grams), which could not be made to crystallize, was fractionated *in vacuo*. The substance boiled at $168-9^\circ$ at 0.7 mm. It crystallized on standing, and, after recrystallization from a little toluene with the aid of ligroin, formed minute prisms softening at 39° and melting at $39.5-40.5^\circ$ (corrected). It gives a faint, pinkish color with sulphuric acid.

0.2589 gm. of substance (Kjeldahl) required 11.40 cc. $\frac{N}{10}$ HCl.

0.2133 gm. of substance required 17.4 cc. $AgNO_3$ Solution II.

Calculated for $C_{11}H_{14}O_2NCl$: N = 6.15 per cent; Cl = 15.57 per cent.

Found: N = 6.17 per cent; Cl = 15.18 per cent.

Chloroacetylaminioethyl o-Tolyl Ether and Hexamethylenetetramine.—Equimolecular amounts of the components were boiled for one hour in dry chloroform. As the salt did not separate, the solution was evaporated to small bulk and treated with dry acetone. This caused the salt to separate, after which the mixture was warmed until the product became entirely crystalline. It melts at 162° to a dark liquid and gives a bright red color with sulphuric acid.

0.1946 gm. of substance required 9.55 cc. $AgNO_3$ Solution II.

Calculated for $C_{17}H_{24}O_2N_3Cl$: Cl = 9.64 per cent.

Found: Cl = 9.12 per cent.

Chloroacetylethylaminioethanol, $ClCH_2CON(C_2H_5)_2C_2H_4OH$.—6 grams of ethylaminoethanol were chloroacetylated in the same manner as was aminoethanol. The reaction mixture was saturated with sodium carbonate and shaken out ten times with chloroform. This was dried and evaporated to small bulk, finally *in vacuo*. Yield of crude product: 5 grams. Roughly three-quarters of this boiled at $130-5^\circ$ at 0.6 mm., yielding a pale yellow, viscous oil which was soluble in and miscible with water.

0.2729 gm. of substance (Kjeldahl) required 16.45 cc. $\frac{N}{10}$ HCl.

0.1967 gm. of substance required 21.3 cc. AgNO₃ Solution II.

Calculated for C₆H₁₂O₂NCl: N = 8.46 per cent; Cl = 21.41 per cent.

Found: N = 8.44 per cent; Cl = 20.14 per cent.

Chloroacetyllethylaminoethyl p-Nitrobenzoate.—As the crude ester did not crystallize it was shaken out from the acid mixture with chloroform. This was washed, first with water, then with dilute sodium carbonate solution, then water, dried, and concentrated to a syrup. This was taken up in a little hot alcohol and allowed to stand, when it gradually crystallized. Recrystallized, with bone-blackening, first from absolute alcohol, then toluene, and finally absolute alcohol, it forms almost colorless, minute rhombs which melt at 73.5–4.° (corrected) with slight preliminary softening. It is difficultly soluble in the cold in ether, absolute alcohol, and toluene, but dissolves readily in benzene and chloroform.

0.1946 gm. of substance required 11.72 cc. AgNO₃ Solution II.

Calculated for C₁₂H₁₈O₂N₂Cl: Cl = 11.27 per cent.

Found: Cl = 11.20 per cent.

Chloroacetyllethylaminoethyl p-Nitrobenzoate and Hexamethylenetetramine.—The chloroform solution, after boiling for one and a half hours, was filtered from a little hexamethylenetetraminium chloride and concentrated to small bulk. The salt separated after the addition of several volumes of dry acetone and scratching. After standing over night it was filtered off and washed with dry acetone. It melts at 154–5° to an orange liquid and is difficultly soluble in water.

0.1394 gm. of substance required 2.9 cc. AgNO₃ Solution I.

Calculated for C₁₈H₂₇O₂N₆Cl: Cl = 7.80 per cent.

Found: Cl = 7.33 per cent.

Chloroacetylphenylaminoethanol, ClCH₂CON(C₆H₅)C₂H₄OH.—20 grams of phenylaminoethanol (oxyethylaniline) were dissolved in benzene and chloroacetylated by means of chloroacetyl chloride in the presence of two-normal sodium hydroxide. The benzene layer was washed successively with water, dilute hydrochloric acid (which removed considerable unchanged amine), and water. After drying, the benzene was evaporated to small bulk and treated with

ligroin, precipitating an oil which gradually crystallized. This was recrystallized twice from warm benzene by adding ligroin until a permanent turbidity appeared. The compound forms prisms which melt at $67.5-8.5^{\circ}$ (corrected) with slight preliminary softening.

0.1998 gm. of substance (Kjeldahl) required 9.14 cc. $\frac{N}{N}$ HCl.

Calculated for $C_{16}H_{15}O_2NCl$: N = 6.56 per cent.

Found: N = 6.41 per cent.

Chloroacetylphenylaminoethyl p-Nitrobenzoate.—The ester was recrystallized three times from absolute alcohol, forming faintly yellow spears which melt at $115-6^{\circ}$ (corrected) with slight preliminary softening. It is rather soluble in benzene, very difficultly in ether.

0.2099 gm. of substance gave 14.85 cc. N (767 mm. and 30.3°).

Calculated for $C_{17}H_{15}O_4N_2Cl$: N = 7.73 per cent.

Found: N = 7.70 per cent.

Chloroacetylphenylaminoethyl p-Nitrobenzoate and Hexamethylenetetramine.—After three hours' boiling, dry acetone was added to the chloroform solution. The salt crystallized slowly on scratching. After several hours in the ice box it was filtered off and washed with dry acetone. Yield: about 40 per cent of the theory. The compound melts at $167-8^{\circ}$ to an orange paste.

0.1998 gm. of substance required 7.45 cc. $AgNO_3$ Solution II.

Calculated for $C_{23}H_{27}O_5N_6Cl$: Cl = 7.05 per cent.

Found: Cl = 6.93 per cent.

Owing to the failure of attempts to obtain crystalline hexamethylenetetraminium salts of chloroacetylphenylaminoethanol and the corresponding iodo-compound the bromoacetyl derivative and several of its esters were prepared. Great difficulty was experienced in obtaining these substances and their hexamethylenetetraminium salts in crystalline form. This applies also to several homologs which we attempted to prepare. The following substance is the only one of this series obtained in a state of purity.

Bromoacetylphenylaminoethanol.—30 grams of phenylaminoethanol were dissolved in 150 cc. of benzene, chilled, and slowly treated with a solution of 22 grams (0.5 mol.) of bromoacetyl bromide in

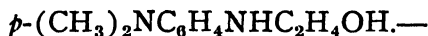
dry benzene. The hydrobromide of the base separated as an oil. The mixture was shaken out successively with dilute hydrochloric acid, water, dilute sodium carbonate solution, and water. After drying, the benzene was evaporated to small bulk and carefully treated with ligroin. The crude bromoacetyl derivative separated on standing and scratching. Yield: 15 grams. When recrystallized from dry ether, rhombic crystals separated first, followed by warty masses. These were mechanically separated from the rhombs and recrystallized from absolute alcohol. This substance forms delicate needles melting at $131-3^{\circ}$ with preliminary softening. It contains bromine, but was not further investigated. The rhombs were recrystallized from dry ether and then melted at $58-60^{\circ}$ (corrected) with slight preliminary softening. These proved to be the desired bromoacetylphenylaminoethanol. It is very soluble in benzene and alcohol and slowly decomposes when exposed to the air.

0.2110 gm. of substance required 15.56 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{10}\text{N}_{12}\text{O}_2\text{NBr}$: Br = 30.97 per cent.

Found: Br = 30.91 per cent.

p-Dimethylaminophenylaminoethanol,



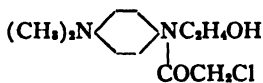
56 grams of *p*-aminodimethylaniline, 32 grams of ethylene chlorohydrin, and 80 cc. of water were boiled for two hours under a reflux. The resulting mixture was made alkaline with sodium hydroxide and shaken out with ether. This was dried, evaporated to small bulk, and fractionated *in vacuo*. 22 grams of the aminoalcohol distilled at $197-204^{\circ}$ at 9 mm. and crystallized on cooling. Of this 14 grams boiled at $215-7^{\circ}$ (corrected) at 16 mm. Recrystallized from toluene, it forms almost colorless aggregates of irregular plates melting at $32.5-5^{\circ}$ (corrected). It is difficultly soluble in toluene at 0° and in ligroin, but dissolves easily in the other organic solvents. It is hygroscopic and dissolves in water. The aqueous solution becomes violet on exposure to the air and gives, with a drop of potassium dichromate solution, a purple color followed by a violet precipitate.

0.2361 gm. of substance (Kjeldahl) required 25.45 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{10}H_{12}ON_2$: N = 15.55 per cent.

Found: N = 15.10 per cent.

Chloroacetyl-p-dimethylaminophenylaminoethanol.—



The base was chloroacetylated in benzene solution by means of chloroacetyl chloride in the presence of two-normal sodium hydroxide solution. The benzene layer was washed with water, dried over sodium sulphate, and evaporated to small bulk. The chloroacetyl derivative crystallized on adding ligroin and scratching. Yield: 40–50 per cent of the theory. Recrystallized twice from absolute alcohol, it forms practically colorless rhombs which melt constantly at $85.5\text{--}7^\circ$ (corrected) with preliminary softening. It is difficultly soluble in cold toluene, alcohol, and ether.

0.0588 gm. of substance gave 5.6 cc. N (768 mm. and 20°).

0.1999 gm. of substance (Carius) gave 0.1109 gm. AgCl.

Calculated for $C_{12}H_{18}O_2N_2Cl$: N = 10.97 per cent; Cl = 13.87 per cent.

Found: N = 10.96 per cent; Cl = 13.72 per cent.

p-Methoxyphenylaminoethanol.—50 grams of *p*-anisidine, 16 grams of ethylene chlorohydrin, and 40 cc. of water were boiled for two hours under a reflux. The resulting mixture was made alkaline with sodium hydroxide and extracted three times with ether. This was rapidly dried over sodium sulphate and evaporated to small bulk. The residue, fractionated *in vacuo*, yielded 17 grams of the aminoalcohol boiling at $187\text{--}97^\circ$ at 8–8.5 mm., of which most boiled at $188\text{--}91^\circ$ at 8 mm. The distillate crystallized in the receiver. A portion recrystallized from dry, alcohol-free ether with the aid of a freezing mixture melted at $43.5\text{--}4.5^\circ$ (corrected).

0.0953 gm. of substance (Kjeldahl) required 5.8 cc. $\frac{N}{10}$ HCl.

Calculated for $C_9H_{11}O_2N$: N = 8.38 per cent.

Found: N = 8.53 per cent.

(3) *Derivatives of γ -Aminopropanol.*

γ -Bromopropyl-p-nitrobenzamide, $BrC_3H_6NHCOC_6H_4NO_2$.—A solution of 18 grams of *p*-nitrobenzoyl chloride in benzene was

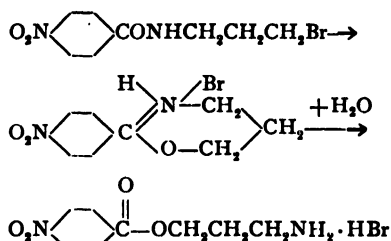
added to a solution of 20 grams of γ -bromopropylamine hydrobromide in 100 cc. of water. With chilling and shaking, 100 cc. of two-normal sodium hydroxide solution were then added in small portions. The amide separated immediately, and was completely precipitated by adding ligroin. Yield: 25 grams. Recrystallized twice from benzene, then once from dry, alcohol-free chloroform, it forms minute, colorless needles which melt at $107.5-8.^{\circ}$ (corrected) with slight preliminary softening. It is soluble in alcohol at room temperature.

0.1741 gm. of substance gave 0.1137 gm. AgBr.

Calculated for $C_{10}H_{11}O_4N_2Br$: Br = 27.83 per cent.

Found: Br = 27.79 per cent.

*γ -Aminopropyl *p*-Nitrobenzoate Hydrobromide.*—20 grams of the above amide were dissolved by heating with water on the water bath. The solution was finally boiled for several hours, completing the rearrangement according to the following scheme:⁸



The resulting solution was concentrated as far as possible on the water bath, forming a solid, crystalline cake on cooling. The hydrobromide was recrystallized from 95 per cent alcohol. Yield: 16 grams. It melts at $185-5.5^{\circ}$ (corrected) and is readily soluble in water.

0.2549 gm. of substance required 8.25 cc. AgNO_3 Solution I.

Calculated for $C_{10}H_{12}O_4N_2 \cdot \text{HBr}$: Br = 26.19 per cent.

Found: Br = 25.67 per cent.

*γ -Oxypropyl-*p*-nitrobenzamide.*—On dissolving γ -aminopropyl *p*-nitrobenzoate hydrobromide in water and adding 1 mol. of sodium hydroxide the free base separates as an oil. This is very unstable,

however, and gradually rearranges to the γ -oxypropyl-*p*-nitrobenzamide, losing its solubility in acids and changing to a mass of heavy, rhombic prisms. Recrystallized from toluene, the amide melts at $102.5-3.5^\circ$ (corrected). It is very difficultly soluble in dry ether and benzene, more soluble in chloroform, and readily so in absolute alcohol.

0.1619 gm. of substance gave 18.1 cc. N (762 mm. and 23.8°).

Calculated for $C_{10}H_{12}O_4N_2$: N = 12.50 per cent.

Found: N = 12.52 per cent.

γ -p-Nitrobenzoylaminopropyl Chloroacetate.—Equimolecular amounts of γ -oxypropyl-*p*-nitrobenzamide and chloroacetic anhydride were dissolved in chloroform and boiled for one hour. The solution was washed first with dilute sodium carbonate solution, then with water, and finally dried. The syrup which remained after concentration crystallized when treated with ligroin and rubbed. Recrystallized first from benzene containing a few drops of toluene, then from absolute alcohol, it forms faintly yellow, crystalline aggregates which melt from 93 to 106° .

0.2512 gm. of substance (Kjeldahl) required 16.6 cc. $\frac{N}{10}$ HCl.

0.1459 gm. of substance required 9.06 cc. $AgNO_3$ Solution II.

Calculated for $C_{12}H_{15}O_5N_2Cl$: N = 9.32 per cent; Cl = 11.80 per cent.

Found: N = 9.26 per cent; Cl = 11.55 per cent.

γ -Chloroacetylaminopropyl p-Nitrobenzoate.—Owing to the rapid rearrangement of γ -aminopropyl *p*-nitrobenzoate, it was found advisable, in order to obtain certain results, to chloroacetylate the base as follows: 5 grams of γ -aminopropyl *p*-nitrobenzoate hydrobromide in 25 cc. of water were chilled in a freezing mixture. To the suspension thus obtained was added a solution of 5 grams of chloroacetyl chloride in 50 cc. of dry, alcohol-free chloroform. The mixture was rapidly turbid, and, with continued cooling, treated drop by drop with 10 per cent sodium hydroxide solution until alkaline. The chloroform layer was then shaken out successively with dilute sodium hydroxide solution, dilute hydrochloric acid, and water. After drying it was concentrated to a syrup which crystallized when treated with ligroin. Recrystallized first from toluene, then twice from absolute alcohol, the ester forms aggregates of flat needles

which melt constantly at $78-9^{\circ}$ (corrected). It is difficultly soluble in ether and hot water, easily at room temperature in alcohol, benzene, and chloroform.

0.1649 gm. of substance gave 14.20 cc. N (765 mm. and 24.5°).

0.1638 gm. of substance required 10.12 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{12}\text{H}_{12}\text{O}_2\text{N}_2\text{Cl}$: N = 9.32 per cent; Cl = 11.80 per cent.

Found: N = 9.65 per cent; Cl = 11.48 per cent.

*γ -Chloroacetylaminopropyl *p*-Nitrobenzoate and Hexamethylenetetramine.*—The salt separates from the chloroform solution. When rapidly heated it melts and decomposes at $178-9^{\circ}$ with preliminary darkening.

0.2000 gm. of substance required 4.55 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{12}\text{H}_{12}\text{O}_2\text{N}_2\text{Cl}$: Cl = 8.05 per cent.

Found: Cl = 8.01 per cent.

*γ -Chloroacetylaminopropyl Anisate (γ -Chloroacetylaminopropyl *p*-Methoxybenzoate).*—11 grams of γ -aminopropyl anisate hydrobromide were dissolved in 100 cc. of water, chilled in a freezing mixture, and treated alternately with small portions of 20 per cent sodium hydroxide solution and chloroacetyl chloride (2 mols.). The mixture was shaken out with chloroform. This was washed as usual, dried, and concentrated. The resulting syrup set to a solid, crystalline cake on adding ligroin and scratching. Yield: 6.5 grams. Recrystallized first from absolute alcohol, then toluene, the ester forms faintly pink, radiating masses of delicate hairs which soften at 72° and melt at $72.5-3^{\circ}$ (corrected). It is rather difficultly soluble in the cold in dry ether and benzene.

0.1309 gm. of substance (Kjeldahl) required 4.65 cc. $\frac{N}{10}$ HCl.

Calculated for $\text{C}_{12}\text{H}_{12}\text{O}_2\text{NCl}$: N = 4.90 per cent.

Found: N = 4.98 per cent.

γ -Chloroacetylaminopropyl Anisate and Hexamethylenetetramine.—After one hour's boiling, dry acetone was added to the chloroform solution, precipitating the salt as glistening leaflets. It melts at $167-8^{\circ}$ to an orange liquid.

0.1993 gm. of substance required 8.65 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{12}\text{H}_{12}\text{O}_2\text{N}_2\text{Cl}$: Cl = 8.33 per cent.

Found: Cl = 8.07 per cent.

(4) *Derivatives of Aminoisopropanol*, $\text{H}_2\text{NCH}_2\text{CH}(\text{CH}_3)\text{OH}$.

Chloroacetylaminoisopropanol, $\text{ClCH}_2\text{CONHCH}_2\text{CH}(\text{CH}_3)\text{OH}$.—12 grams of aminoisopropyl alcohol⁹ were chloroacetylated in the same way as the previous aliphatic aminoalcohols. Solid potassium carbonate was added to the reaction mixture until the chloroacetyl derivative separated as an oil. This was shaken out with chloroform and the aqueous solution extracted nine times more. The residual oil weighed 20 grams. This was fractionated *in vacuo*. The main portion boiled at $131-2^\circ$ (corrected) at 0.6 mm. and crystallized on standing. It melts at $33-4.5^\circ$ (corrected).

0.2462 gm. of substance (Kjeldahl) required 16.25 cc. $\frac{N}{10}$ HCl.

0.1560 gm. of substance required 19.45 cc. AgNO_3 Solution II.

Calculated for $\text{C}_6\text{H}_{10}\text{O}_2\text{NCl}$: N = 9.24 per cent; Cl = 23.39 per cent.

Found: N = 9.24 per cent; Cl = 23.18 per cent.

Chloroacetylaminoisopropanol and Hexamethylenetetramine.—The salt separates from the boiling chloroform solution. It decomposes at $171-2^\circ$.

0.2036 gm. of substance required 13.3 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{11}\text{H}_{22}\text{O}_2\text{N}_2\text{Cl}$: Cl = 12.16 per cent.

Found: Cl = 12.15 per cent.

Chloroacetylaminoisopropyl p-Nitrobenzoate.—Recrystallized from absolute alcohol, in which it is very sparingly soluble in the cold, the ester forms very faintly yellow, silky needles which melt constantly at $133.5-4.5^\circ$ (corrected) with slight preliminary softening. It is soluble in cold acetone, but sparingly so in cold toluene.

0.1418 gm. of substance gave 11.9 cc. N (756 mm. and 23°).

Calculated for $\text{C}_{12}\text{H}_{18}\text{O}_4\text{N}_2\text{Cl}$: N = 9.32 per cent.

Found: N = 9.36 per cent.

Chloroacetylaminoisopropyl p-Nitrobenzoate and Hexamethylenetetramine.—After boiling the dry chloroform solution of the

⁹ Prepared by hydrolyzing β -oxypropylbenzamide (P. Hirsch: *Ber. d. deutsch. chem. Gesellsch.*, xxiii, p. 970, 1890; Gabriel and Heymann: *loc. cit.*) in a sealed tube with 1:1 hydrochloric acid at 150° for four hours. The benzoic acid was filtered off and the filtrate evaporated to dryness, taken up in water, and saturated with potassium carbonate. The amine was drawn off, dried over potassium carbonate, and fractionated.

components for one hour, most of the chloroform was evaporated off. The addition of dry acetone to the residual solution resulted in the precipitation of a mixture of the salt and unchanged hexamethylenetetramine. This was boiled with a small amount of dry chloroform in order to remove the base and was then washed with dry chloroform and dry acetone. Yield: about 35 per cent of the theory. The salt forms very faintly yellow plates which darken above 170° and melt with decomposition at $180-2^{\circ}$. It is somewhat sparingly soluble in water.

0.1743 gm. of substance required 7.30 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{10}\text{H}_{12}\text{O}_4\text{N}_4\text{Cl}$: Cl = 8.05 per cent.

Found: Cl = 7.79 per cent.

Aminoisopropyl p-Nitrobenzoate Hydrobromide.—*p*-Nitrobenzoyl-aminoisopropyl bromide¹⁰ was covered with hot water and boiled until completely dissolved. The solution was then concentrated as far as possible on the water bath. On cooling, the residue set to a solid cake which was recrystallized from alcohol. It melts at $221-2^{\circ}$ and is rather difficultly soluble in cold water. The salt was analyzed for amino nitrogen by the Van Slyke method.

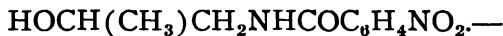
0.2004 gm. of substance gave 16.5 cc. N (761 mm. and 22°).

0.1993 gm. of substance required 6.5 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{10}\text{H}_{12}\text{O}_4\text{N}_4 \cdot \text{HBr}$: Amino N = 4.59%; Br = 26.19%.

Found: Amino N = 4.65%; Br = 25.85%.

Oxyisopropyl-p-nitrobenzamide,



When alkali is added to the aqueous solution of the above hydrobromide a transitory cloudiness appears, followed by the deposition of glistening platelets melting at $168-9^{\circ}$. This is not the free aminoisopropyl *p*-nitrobenzoate, as claimed by Uedinck,¹⁰ but is oxyisopropyl-*p*-nitrobenzamide. It thus appears that the rearrangement takes place with greater rapidity in this case than in that of the previously discussed normal propyl isomers. The correctness of this interpretation is shown by the insolubility of the substance in dilute acids, by the fact that it contains no amino nitrogen, as deter-

¹⁰ A. Uedinck: *Ber. d. deutsch. chem. Gesellsch.*, xxxii, p. 978, 1899.

mined by Van Slyke's method, and by the fact that it forms a chloroacetate (see below), not a chloroacetyl amino derivative, as is shown by the absence of *p*-nitrobenzoic acid in the saponification products after gentle treatment with alcoholic sodium hydroxide, and by direct comparison with chloroacetyl aminoisopropyl *p*-nitrobenzoate (see above).

p-Nitrobenzoylaminoisopropyl Chloroacetate,



13 grams of the above oxyisopropyl-*p*-nitrobenzamide were suspended in a small volume of dry, alcohol-free chloroform and treated with 7 grams of chloroacetyl chloride. The mixture was boiled under a reflux until a clear solution had been obtained and the evolution of hydrochloric acid gas had ceased. More chloroform was added and the mixture shaken out with dilute sodium hydroxide solution. After drying and concentrating, the substance was obtained in crystalline form by the addition of ligroin. Yield: 13 grams. Recrystallized first from absolute alcohol, then twice from toluene, and finally from absolute alcohol, the ester forms almost colorless plates and prisms melting at 89–94° (corrected). The ester is soluble in cold ether and acetone.

0.1447 gm. of substance (Kjeldahl) required 9.80 cc. $\frac{N}{10}$ HCl.

0.1509 gm. of substance required 9.27 cc. AgNO₃ Solution II.

Calculated for C₁₃H₁₅O₅N₂Cl: N = 9.32 per cent; Cl = 11.80 per cent.

Found: N = 9.49 per cent; Cl = 11.42 per cent.

p-Nitrobenzoylaminoisopropyl Chloroacetate and Hexamethylene-tetramine.—The salt separates from the hot chloroform solution as an oil which redissolves on cooling. It was obtained crystalline by adding several volumes of dry acetone and scratching. It melts with decomposition at 175–8°.

0.2381 gm. of substance required 5.55 cc. AgNO₃ Solution I.

Calculated for C₁₈H₂₈O₅N₆Cl: Cl = 8.05 per cent.

Found: Cl = 8.21 per cent.

(5) *Derivatives of the Higher Aliphatic Aminoalcohols.*

δ-Chloroacetyl amino-*n*-butanol, ClCH₂CONHC₄H₉OH.—This was prepared from δ-aminobutanol¹¹ in the same way as the isopro-

¹¹ L. Henry: *Ber. d. deutsch. chem. Gesellsch.*, xxxiii, p. 3170, 1900.

pyl homolog. The reaction mixture, after addition of potassium carbonate, was shaken out six times with chloroform. This was dried, evaporated, and concentrated to a syrup. Yield, from 12 grams of aminoalcohol: 19 grams. When fractionated *in vacuo*, the main portion boiled at $165-7^{\circ}$ (corrected) at 0.7 mm. and crystallized on cooling. It melts at 30° (corrected) and is soluble in water, alcohol, and chloroform, but very sparingly so in benzene.

0.3001 gm. of substance (Kjeldahl) required 18.2 cc. $\frac{N}{10}$ HCl.

0.2465 gm. of substance required 28.56 cc. AgNO_3 Solution II.

Calculated for $\text{C}_4\text{H}_{12}\text{O}_2\text{NCl}$: N = 8.46 per cent; Cl = 21.41 per cent.

Found: N = 8.49 per cent; Cl = 21.55 per cent.

δ -Chloroacetyl-amino-*n*-butanol and Hexamethylenetetramine.

The salt gradually separated from the chloroform solution as an oil. On adding an equal volume of dry acetone and allowing to stand protected from moisture it gradually crystallized. The salt is hygroscopic. It sinters at 122° and melts at 125° .

0.2273 gm. of substance required 13.6 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{12}\text{H}_{24}\text{O}_2\text{N}_6\text{Cl}$: Cl = 11.61 per cent.

Found: Cl = 11.13 per cent.

δ -Chloroacetylaminobutyl *p*-Nitrobenzoate.—The ester was recrystallized first from absolute alcohol, then toluene, forming practically white rosettes of minute prisms melting at 79° (corrected). It is soluble in the cold in ether and benzene, and extremely so in chloroform.

0.1489 gm. of substance gave 11.8 cc. N (760 mm. and 22.5°).

Calculated for $\text{C}_{12}\text{H}_{18}\text{O}_4\text{N}_2\text{Cl}$: N = 8.91 per cent.

Found: N = 8.91 per cent.

δ -Chloroacetylaminobutyl *p*-Nitrobenzoate and Hexamethylenetetramine.—After one and one-half hours' boiling, the salt crystallized from the chloroform solution on cooling and scratching. It forms thick, faintly yellow plates which darken above 165° and melt to a thick, brown liquid at $170-2^{\circ}$. It is sparingly soluble in water.

0.1735 gm. of substance required 7.01 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{12}\text{H}_{18}\text{O}_4\text{N}_2\text{Cl}$: Cl = 7.80 per cent.

Found: Cl = 7.51 per cent.

β-Chloroacetyl-amino-γ-butanol,

β-Amino-γ-butanol (made by the reduction of methyl isonitrosoethyl ketone¹²) was chloroacetylated and isolated in the same way as in the previous cases. The crude product crystallized partially, but was purified by distillation *in vacuo*. It boils at 119° (corrected) at 0.3 mm. and solidifies on cooling to a crystalline mass which melts at 38–9° (corrected). It has a faint isonitrile-like odor, and is less readily soluble in benzene than in water, alcohol, and chloroform.

0.2397 gm. of substance (Kjeldahl) required 14.35 cc. $\frac{N}{10}$ HCl.

0.1017 gm. of substance required 11.63 cc. AgNO₃ Solution II.

Calculated for C₆H₁₂O₂NCl: N = 8.46 per cent; Cl = 21.41 per cent.

Found: N = 8.39 per cent; Cl = 21.27 per cent.

β-Chloroacetyl-amino-γ-butanol and Hexamethylenetetramine.—

The salt separates from the boiling chloroform solution. It melts at 167–9°.

0.2100 gm. of substance required 13.4 cc. AgNO₃ Solution II.

Calculated for C₁₂H₂₄O₂N₆Cl: Cl = 11.61 per cent.

Found: Cl = 11.87 per cent.

*β-Chloroacetyl-amino-γ-butyl p-Nitrobenzoate.—*Recrystallized first from absolute alcohol, then toluene, the ester forms pale cream-colored rosettes of delicate leaflets which melt at 117–8° (corrected). It dissolves readily in cold acetone and chloroform, more sparingly, however, in ether. Yield: about 40 per cent of the theory.

0.1485 gm. of substance gave 11.8 cc. N (755 mm. and 21.5°).

Calculated for C₁₈H₁₈O₄N₂Cl: N = 8.91 per cent.

Found: N = 8.92 per cent.

*β-Chloroacetyl-amino-γ-butyl p-Nitrobenzoate and Hexamethylenetetramine.—*The salt separates from the boiling chloroform solution of the components. It forms colorless micro-crystals which melt with decomposition at 189–91° and dissolve readily in water. Yield: about 45 per cent of the theory.

¹² E. Strauss: *Ber. d. deutsch. chem. Gesellsch.*, xxxiii, p. 2827, 1900.

0.1689 gm. of substance required 6.83 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{10}\text{H}_{17}\text{O}_2\text{N}_4\text{Cl}$: $\text{Cl} = 7.80$ per cent.

Found: $\text{Cl} = 7.52$ per cent.

γ -Chloroacetyl-amino- β -pentanol,



5 grams of γ -amino- β -pentanol (prepared by reduction of methyl isonitrosopropyl ketone according to Jänecke's method¹³) were chloroacetylated in the usual manner. The crude product crystallized on cooling. Yield: 8 grams. It boils at $126-8^\circ$ at 0.3 mm. and crystallizes on cooling to a waxy solid which melts at $52-60^\circ$. No suitable means of recrystallization could be found. The compound is somewhat hygroscopic and is easily soluble in water, alcohol, acetone, and chloroform, difficultly in benzene and ligroin.

0.2463 gm. of substance (Kjeldahl) required 14.05 cc. $\frac{N}{10}$ HCl .

Calculated for $\text{C}_7\text{H}_{14}\text{O}_2\text{NCl}$: $\text{N} = 7.80$ per cent.

Found: $\text{N} = 7.99$ per cent.

γ -Chloroacetyl-amino- β -pentanol and Hexamethylenetetramine.—

Addition of the components proceeded slowly in chloroform solution. Several volumes of dry acetone were finally added, completing the precipitation. The salt was boiled out with dry acetone and dried. It melts and decomposes at $167-8^\circ$ with preliminary darkening and is somewhat hygroscopic.

0.1932 gm. of substance required 12.1 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{10}\text{H}_{18}\text{O}_2\text{N}_6\text{Cl}$: $\text{Cl} = 11.09$ per cent.

Found: $\text{Cl} = 11.65$ per cent.

Chloroacetylaminomethylmethylethyl Carbinol (α -Chloroacetyl-amino- β -methyl- β -butanol), $\text{C}_2\text{H}_5\text{C}(\text{OH})\text{CH}_2\text{NHCOCH}_2\text{Cl}.$ —
 $\begin{array}{c} | \\ \text{CH}_3 \end{array}$

Aminomethylmethylethyl carbinol¹⁴ was chloroacetylated in the usual manner. The crude product was fractionated *in vacuo*, the portion taken boiling at 134° (corrected) at 0.7 mm. It was not

¹³ E. Jänecke: *Ber. d. deutsch. chem. Gesellschaft.*, xxxii, p. 1095, 1899.

¹⁴ D. R.-P. 189481; *Friedlaenders Fortschr. d. Teerfarbenfabrikation*, viii, p. 1038, 1905-07.

obtained crystalline. It is soluble in water, alcohol, ether, acetone, chloroform, and benzene.

0.2600 gm. of substance (Kjeldahl) required 14.55 cc. $\frac{N}{10}$ HCl.

0.1700 gm. of substance required 17.75 cc. AgNO₃ Solution II.

Calculated for C₇H₁₁O₂NCl: N = 7.80 per cent; Cl = 19.74 per cent.

Found: N = 7.84 per cent; Cl = 19.41 per cent.

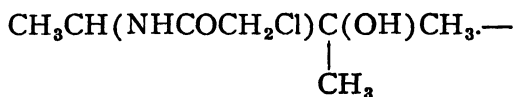
Chloroacetylaminomethylmethylethyl Carbinol and Hexamethylenetetramine.—The salt separated from the boiling chloroform solution. It melts with decomposition at 170° and is somewhat hygroscopic.

0.2240 gm. of substance required 13.8 cc. AgNO₃ Solution II.

Calculated for C₁₁H₂₀O₂N₂Cl: Cl = 11.09 per cent.

Found: Cl = 11.46 per cent.

γ-Chloroacetyl-amino-β-methyl-β-butanol,



15 grams of γ-amino-β-methyl-β-butanol¹⁵ were chloroacetylated in the usual manner. The reaction mixture was neutralized and shaken out ten times with chloroform. The crude residue from the chloroform extract weighed 19 grams. When fractionated *in vacuo*, the main portion boiled at 122° (corrected) at 0.4 mm. and did not crystallize on cooling. It is soluble in water, alcohol, acetone, chloroform, and ether. It is miscible with a small amount of benzene but separates again when a larger quantity is added.

0.2148 gm. of substance (Kjeldahl) required 12.05 cc. $\frac{N}{10}$ HCl.

0.2235 gm. of substance required 23.22 cc. AgNO₃ Solution II.

Calculated for C₇H₁₁O₂NCl: N = 7.80 per cent; Cl = 19.74 per cent.

Found: N = 7.88 per cent; Cl = 19.33 per cent.

γ-Chloroacetyl-amino-β-methyl-β-butanol and Hexamethylenetetramine.—The product suddenly separated from the boiling chloroform solution. It melts with decomposition at 174–5°.

0.2285 gm. of substance required 13.2 cc. AgNO₃ Solution II.

Calculated for C₁₁H₂₀O₂N₂Cl: Cl = 11.10 per cent.

Found: Cl = 10.75 per cent.

¹⁵ K. Krassousky: *Compt. rend. Acad. d. sc.*, cxlvi, p. 237, 1908.

(6) *C-Aryl Derivatives of Aminoalcohols.* *α -Phenyl- α -oxy- β -chloroacetylaminioethane,*

14.7 grams of α -phenyl- α -oxyethylamine were obtained by reducing 35 grams of benzaldehyde cyanohydrin in 50 per cent alcohol solution with 1400 grams of 4 per cent sodium amalgam, keeping the mixture just acid with acetic acid.¹⁶ The crude product was suspended in 85 cc. of two-normal sodium hydroxide solution and diluted with enough water to dissolve most of the amine. 11 cc. of chloroacetyl chloride were then added in small amounts, with cooling and vigorous shaking. Most of the chloroacetyl derivative separated as an oil, an additional quantity being salted out by means of potassium carbonate. The mixture was extracted five times with chloroform. This was washed successively with dilute hydrochloric acid, water, and sodium carbonate solution. The chloroform solution, after drying over potassium carbonate, was evaporated to small bulk. The residue crystallized while still hot and was recrystallized from benzene containing a little ligroin. Yield: 10.2 grams. Recrystallized again from chloroform, it forms thick plates which soften at 108° and melt at 109–9.5° (corrected). It is soluble in hot water, hot chloroform, very difficultly in the cold in dry ether and chloroform, but easily in acetone.

0.2193 gm. of substance (Kjeldahl) required 10.35 cc. $\frac{N}{10}$ HCl.

Calculated for $\text{C}_{10}\text{H}_{12}\text{O}_2\text{NCl}$: N = 6.56 per cent.

Found: N = 6.61 per cent.

α -Phenyl- α -oxy- β -chloroacetylaminioethane and Hexamethylenetetramine.—The salt separates from the boiling chloroform solution. It melts at 179° with decomposition.

0.1652 gm. of substance required 4.8 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{10}\text{H}_{12}\text{O}_2\text{N}_6\text{Cl}$: Cl = 10.02 per cent.

Found: Cl = 10.23 per cent.

α -p-Tolyl- α -oxyethylamine, $\text{CH}_3\text{C}_6\text{H}_4\text{CH}(\text{OH})\text{CH}_2\text{NH}_2$.—35

¹⁶ Compare D. R.-P. 193634; *Friedlaender's Fortschr. d. Teerfarbenfabrikation*, viii, p. 1183, 1905–07.

grams of crude *p*-tolualdehyde cyanohydrin (from the aldehyde bisulphite and potassium cyanide) were dissolved in 550 cc. of 50 per cent alcohol, cooled in a freezing mixture, and, with vigorous turbinizing, reduced with 1230 grams of 4 per cent sodium amalgam. During the process, which lasted several hours, the mixture was kept slightly acid with acetic acid.¹⁶ The solution was then evaporated to about one-half volume. The solid mass of salts obtained on cooling was broken up, diluted with a little dilute hydrochloric acid, and filtered. A small amount of a semi-solid by-product remained with the salts. The filtrate was shaken out with ether to remove other impurities and was then covered with ether and made strongly alkaline with sodium hydroxide. After extracting four times with ether the ethereal solution was rapidly dried over potassium hydroxide and evaporated to small bulk. On cooling, the residue crystallized. Bone-blackened and recrystallized from benzene, the amine forms snow-white crystals which melt at 68–9° (corrected). Yield: 9.1 grams. It is difficultly soluble in ether and cold benzene, easily in alcohol and water.

0.2160 gm. of substance (Kjeldahl) required 14.05 cc. $\frac{N}{10}$ HCl.

Calculated for $C_9H_{10}ON$: N = 9.27 per cent.

Found: N = 9.11 per cent.

α -p-Tolyl- α -oxy- β -chloroacetylaminooethane.—8.2 grams of the amine were chloroacetylated as in the case of the phenyl compound. The oily chloroacetyl derivative crystallized in the reaction mixture when seeded with a few crystals obtained from a test portion in 50 per cent alcohol by cooling in a freezing mixture. Yield: 5.1 grams after recrystallization from 50 per cent alcohol as above. Recrystallized again from benzene containing a little ligroin, it forms thick, colorless plates or prisms which melt at 81–2° (corrected) with slight preliminary softening. It is readily soluble in ether and chloroform.

0.2087 gm. of substance (Kjeldahl) required 9.4 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{11}H_{14}O_2NCl$: N = 6.16 per cent.

Found: N = 6.31 per cent.

α -p-Methoxyphenyl- α -oxyethylamine.—24 grams of anisaldehyde cyanohydrin (prepared from the aldehyde according to D. R.-P.

85230¹⁷) were reduced as in the previous examples. The solution was evaporated to about one-half volume, made more strongly acid with hydrochloric acid, and extracted twice with ether to remove impurities. After filtering from a small amount of insoluble material the aqueous solution was covered with ether and made strongly alkaline. After three extractions with ether this was rapidly dried over potassium hydroxide and evaporated to small bulk. The residue crystallized on cooling and was exhausted *in vacuo*. Yield: 6 grams. For analysis, a small portion was recrystallized from a mixture of benzene and ligroin.

0.1893 gm. of substance (Kjeldahl) required 10.75 cc. $\frac{N}{10}$ HCl.

Calculated for $C_8H_{10}O_2N$: N = 8.38 per cent.

Found: N = 7.95 per cent.

α, β -Diphenylchloroacetylamin ethanol,



To a boiling solution of 5.6 grams of α, β -diphenylaminoethanol in dry benzene was added, drop by drop, a solution of 4.5 grams of chloroacetic anhydride in dry benzene. The solution was boiled for one hour, being allowed to concentrate at the same time to a volume of about 50 cc. The chloroacetyl derivative separated on cooling. The separation was completed by adding about two volumes of ligroin. After filtering, the product was recrystallized from absolute alcohol, forming interlaced, hair-like needles. Yield: 3.8 grams. It melts at 194–4.5° (corrected) and is difficultly soluble in cold absolute alcohol, chloroform, benzene, and ethyl acetate, but easily in acetone. It gives an olive-yellow color with sulphuric acid.

0.2907 gm. of substance (Kjeldahl) required 10.15 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{18}H_{16}O_2NCl$: N = 4.84 per cent.

Found: N = 4.89 per cent.

α, β -Diphenylchloroacetylamin ethanol and Hexamethylenetetramine.—The components reacted very slowly in boiling chloroform

¹⁷ D. R.-P. 85230; *Friedlaenders Fortschr. d. Teerfarbenfabrikation*, iv, p. 160, 1894–97.

solution. After two hours the product was filtered off, washed with dry chloroform and dry acetone, and boiled for one hour with dry chloroform to complete the removal of the unchanged chloroacetyl derivative. Yield: poor. The salt forms hexagonal platelets containing solvent of crystallization which is lost on drying at 100° *in vacuo*. The compound then melts at 174° with decomposition. It gives a brown-orange color with sulphuric acid.

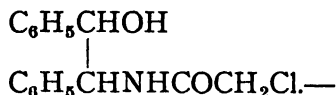
0.1991 gm. of substance required 4.54 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{22}\text{H}_{20}\text{O}_2\text{N}_2\text{Cl}$: $\text{Cl} = 8.25$ per cent.

Found: $\text{Cl} = 8.02$ per cent.

The salt was obtained in better yield by heating the components with dry chloroform in a pressure bottle at 100° for one and one-quarter hours, but then required several hours' boiling with successive portions of dry acetone to free it from a water-insoluble impurity. After this treatment the salt contained acetone of crystallization, as was shown by the iodoform test. This was given off at 100° *in vacuo*.

α, β -Isodiphenylchloroacetylamin ethanol,



10 grams of α, β -isodiphenylaminoethanol¹⁸ were chloroacetylated in the same way as its isomer. During the addition of the chloroacetic anhydride a voluminous precipitate separated. This went into solution on adding more benzene and continuing the addition of the anhydride to the boiling solution. The mixture was then boiled for three-quarters of an hour, being concentrated at the same time. The crystals which separated on standing over night were filtered off and recrystallized from benzene and then from absolute alcohol. Yield: 2.5 grams. The compound forms colorless needles which melt at $156-7^{\circ}$ (corrected) and are difficultly soluble in ether, readily in chloroform. It gives a greenish yellow color with sulphuric acid.

¹⁸ E. Erlenmeyer, Jr.: *Ann. d. Chem.*, cccvii, p. 119, 1899; cccxxxvii, p. 320, 1904.

0.2463 gm. of substance (Kjeldahl) required 8.6 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{10}H_{16}O_2NCl$: N = 4.84 per cent.

Found: N = 4.88 per cent.

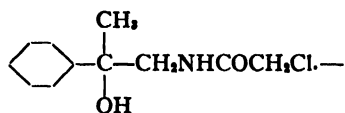
α , β -Isodiphenylchloroacetylaminopropane and Hexamethylenetetramine.—The solution of the components in dry, boiling chloroform set to a thick cake after fifteen minutes. This was broken up and the boiling continued for one-half hour, after which the salt was filtered off and washed well with dry chloroform and dry acetone. An additional quantity was obtained by boiling the filtrate from the first crop. The salt is very difficultly soluble in water and gives a straw color with sulphuric acid. It melts and decomposes at $197-9^\circ$.

0.2317 gm. of substance required 5.28 cc. $AgNO_3$ Solution I.

Calculated for $C_{22}H_{20}O_2N_2Cl$: Cl = 8.25 per cent.

Found: Cl = 8.02 per cent.

β -Phenyl- β -Oxy- α -Chloroacetylaminopropane,



92 grams of β -phenyl- β -oxy- α -chloropropane¹⁹ were converted into the amino compound according to D. R.-P. 183361²⁰ and 189481,¹⁴ except that the resulting mixture of bases was worked up by the usual methods instead of by the procedure given in the patents. Yield of β -phenyl- β -oxy- α -aminopropane: 28 grams, boiling at $133-40^\circ$ at 14 mm. 14 grams of the amine were dissolved in benzene and chloroacetylated in the usual manner with chloroacetyl chloride in the presence of two-normal sodium hydroxide. Ether was added and the upper layer shaken out successively with 5 per cent aqueous hydrochloric acid, water, dilute sodium carbonate solution, and water. After drying over sodium sulphate the solvents were evaporated off and the hot residue was exhausted *in vacuo*. 15.7 grams of the chloroacetyl derivative were obtained as a viscous oil which could not be made to crystallize.

¹⁹ M. Tiffeneau: *Compt. rend. Acad. d. sc.*, cxxxiv, p. 775, 1902.

²⁰ D. R.-P. 183361; *Friedlaenders Fortschr. d. Teerfarbenfabrikation*, viii, p. 1041, 1905-07.

β -Phenyl- β -oxy- α -chloroacetylaminopropane and Hexamethylene-tetramine.—The salt separates rapidly from the boiling chloroform solution of the components. It darkens slightly above 165° and melts with decomposition at 175–6°.

0.1972 gm. of substance required 9.96 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{17}\text{H}_{20}\text{O}_2\text{N}_6\text{Cl}$: Cl = 9.64 per cent.

Found: Cl = 9.39 per cent.

α -Phenyl- α -benzoyloxy- β -benzoylaminopropane,



In the course of a series of uncompleted experiments α -phenyl- α -oxy- β -aminopropane was prepared by the reduction of isonitrosopropiophenone with sodium amalgam. As the crude product so obtained melted 10 degrees below the melting point assigned by Rabe and Hunnius,²¹ it was sought to identify the aminoalcohol by the preparation of some characteristic derivative. To this end 0.7 gram was dissolved in pyridine and treated in the cold with 3 mols. of benzoyl chloride. After standing for six hours the mixture was poured into water, precipitating an oil which soon solidified. After two recrystallizations from absolute alcohol, the dibenzoyl derivative formed minute, glistening prisms melting at 172.5–3.5° (corrected) with slight preliminary softening. Yield: 0.8 gram. It is readily soluble in acetone.

0.2972 gm. of substance (Kjeldahl) required 8.10 cc. $\frac{N}{10}$ HCl.

Calculated for $\text{C}_{28}\text{H}_{21}\text{O}_4\text{N}$: N = 3.90 per cent.

Found: N = 3.82 per cent.

The chloroacetyl derivative of α -phenyl- α -oxy- β -aminopropane was prepared, but formed an uncrystallizable oil which decomposed partially when distilled in a high vacuum.

²¹ P. Rabe and T. Hunnius: *Ber. d. deutsch. chem. Gesellsch.*, xlv, p. 2166, 1912.

THE QUATERNARY SALTS OF HEXAMETHYLENE- TETRAMINE.

VI. HALOGENETHYL ETHERS AND ESTERS AND THEIR HEXAMETHYLENETETRAMINIUM SALTS.

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As far as the authors are aware, the literature contains no reference to the use of the halogenethyl ethers and esters for the preparation of quaternary salts of hexamethylenetetramine. In the present work the few chloroethyl compounds tested failed to react with the base, so recourse was had to the bromoethyl compounds. These were preferred to the iodo derivatives, since, as often observed with other types of halogen compounds, the salts resulting from the iodo derivatives proved to be insoluble in water.

With both the bromoethers and esters the reaction with hexamethylenetetramine was found to be very slow and in most cases the yields were poor. The best results were obtained by the use of a high boiling solvent such as toluene or by allowing the chloroform solution of the components to stand at room temperature for several months. The low solubility of hexamethylenetetramine in boiling toluene and the danger of decomposition at this high temperature are disadvantages of the former method.

During the work with this group of substances, occasion demanded the preparation of a number of new bromoethyl ethers and esters, a description of which will be found in the following.

EXPERIMENTAL.

(1) *Bromoethyl Ethers and Their Derivatives.*

Phenoxyethylhexamethylenetetraminium Bromide.—The salt gradually separated in small yield from the solution of phenoxyethyl

bromide and the base in dry chloroform during several hours' boiling. Additional amounts were obtained by further heating of the filtrate. The salt melts at $151-2^{\circ}$, is easily soluble in water, and gives a faint pink color with sulphuric acid.

0.2002 gm. of substance required 6.0 cc. AgNO_3 Solution I.¹

Calculated for $\text{C}_{14}\text{H}_{21}\text{ON}_4\text{Br}$: Br = 23.42 per cent.

Found: Br = 23.78 per cent.

o-Methylphenoxyethylhexamethylenetetraminium Bromide.—22 grams of *o*-cresoxyethyl bromide² and 15 grams of hexamethylenetetramine were dissolved in 150 cc. of dry chloroform and allowed to stand five months at room temperature. Yield: 30 grams of salt, melting at 185° . It forms glistening prisms and plates which dissolve with difficulty in water. It turns pink in sulphuric acid, giving an orange solution which gradually changes to red.

0.2091 gm. of substance required 5.9 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{15}\text{H}_{23}\text{ON}_4\text{Br}$: Br = 22.49 per cent.

Found: Br = 22.38 per cent.

m-Methylphenoxyethyl Bromide (*m*-Cresoxyethyl Bromide).—108 grams of *m*-cresol, a solution of 80 grams of 50 per cent aqueous sodium hydroxide in 250 cc. of absolute alcohol, and 300 grams of ethylene bromide were boiled three to four hours under a reflux. The mixture was then diluted with water, made distinctly alkaline with sodium hydroxide, and extracted with ether. The ether was distilled off and the residue fractionated *in vacuo*. The bromoethyl ether was obtained as a colorless oil boiling at $136-7^{\circ}$ at 14 mm. Yield: 55 grams. This was redistilled before analysis.

0.1539 gm. of substance (Carius) gave 0.1314 gm. AgBr .

Calculated for $\text{C}_9\text{H}_{11}\text{OBr}$: Br = 37.16 per cent.

Found: Br = 36.34 per cent.

The high-boiling residue from the distillation of the above bromoethyl ether was taken up in hot alcohol and filtered. The ethylene ether separated on cooling, but was not further investigated.

m-Methylphenoxyethylhexamethylenetetraminium Bromide.—This was prepared in the same way as the *ortho* isomer. The salt

¹ 1 cc. = 0.00352 gm. Cl; 0.00793 gm. Br; 0.01259 gm. I.

² L. Gattermann: *Ann. d. Chem.*, ccclvii, p. 356, 1907.

separated with chloroform of crystallization, and was therefore boiled with dry acetone. As it was still found to be impure, it was taken up in hot absolute alcohol and filtered rapidly from the undissolved residue. 5 grams of the salt separated on cooling. It melted at $155-6^{\circ}$, dissolved readily in water, and gave a pink color with sulphuric acid.

0.1779 gm. of substance required 5.15 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{10}\text{H}_{12}\text{ON}_4\text{Br}$: Br = 22.49 per cent.

Found: Br = 22.92 per cent.

p-Methylphenoxyethylhexamethylenetetraminium Bromide.—The salt was obtained in the same way as the *ortho* isomer. Yield: 33 grams, containing chloroform of crystallization. After boiling with dry acetone, the salt melted at 176° . It was somewhat more readily soluble in water than the *ortho* isomer, and gave no color with sulphuric acid.

0.2071 gm. of substance required 5.8 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{10}\text{H}_{12}\text{ON}_4\text{Br}$: Br = 22.49 per cent.

Found: Br = 22.21 per cent.

α -Naphthyl Bromoethyl Ether (α -Naphthoxyethyl Bromide).—This was prepared in the same way as *m*-cresoxyethyl bromide. Yield: 110 grams, boiling at $154-6^{\circ}$ at 0.8 mm., from 145 grams of α -naphthol, 80 grams of 50 per cent aqueous sodium hydroxide in 250 cc. of absolute alcohol, and 500 grams of ethylene bromide. The boiling point was unchanged by a subsequent distillation. A few drops of the substance, on long standing in a freezing mixture, with occasional rubbing, yielded crystals which caused the solidification of the entire distillate. Recrystallized from ligroin with the aid of a freezing mixture, the ether forms transparent, hexagonal plates which melt at 25° (corrected) with slight preliminary softening. At room temperature it is sparingly soluble in methyl and ethyl alcohols, readily so in benzene, ether, and ligroin. It turns orange under sulphuric acid and dissolves with a greenish yellow color.

0.2253 gm. of substance gave 0.1657 gm. AgBr .

Calculated for $\text{C}_{12}\text{H}_{11}\text{OBr}$: Br = 31.83 per cent.

Found: Br = 31.29 per cent.

Practically no ethylene ether was formed in this experiment.

α -Naphthoxyethylhexamethylenetetraminium Bromide.—This was prepared in the same manner as the three preceding salts. The compound forms glistening platelets which turn yellow above 170° , melt with decomposition at about 175° , and are very difficultly soluble in water. With sulphuric acid it gives a transitory green color which changes to brown by reflected light and blue by transmitted light.

0.2042 gm. of substance required 5.3 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{18}\text{H}_{23}\text{ON}_4\text{Br}$: Br = 20.38 per cent.

Found: Br = 20.58 per cent.

β -Naphthoxyethylhexamethylenetetraminium Bromide.—The salt, prepared as in the above cases, separated in glistening leaflets containing chloroform of crystallization which could not be removed by boiling with acetone or alcohol. It is very difficultly soluble in water, gives an olive-yellow color with sulphuric acid, and decomposes at about 180° . An aqueous suspension, when boiled, evolves chloroform and acquires a nerolin-like odor.

0.2075 gm. of substance required 8.87 cc. AgNO_3 Solution II.³

Calculated for $\text{C}_{18}\text{H}_{23}\text{ON}_4\text{Br} \cdot \frac{1}{2}\text{CHCl}_3$: Br = 17.71 per cent.

Found: Br = 17.91 per cent.

2,4,6-Trichlorophenoxyethyl Bromide.—50 grams of 2,4,6-trichlorophenol were dissolved in 100 cc. of absolute alcohol containing 20 grams of 50 per cent aqueous sodium hydroxide and mixed with 300 grams of ethylene bromide. The mixture was boiled for five hours, separating into two layers. After adding an excess of sodium hydroxide, the unused ethylene bromide was blown off with steam. On cooling, the oily reaction product crystallized. This was shaken out with chloroform, and, on evaporation of the solvent, remained in practically pure form. Recrystallized twice from absolute alcohol, it melts at $47-8^{\circ}$ (corrected). It is readily soluble in ether, benzene, and toluene, difficultly in cold absolute alcohol.

0.1828 gm. of substance (Carius) gave 0.3707 gm. $\text{AgCl} + \text{AgBr}$.

Calculated for $\text{C}_6\text{H}_3\text{OCl}_3\text{Br}$: Cl = 34.93 per cent; Br = 26.24 per cent.

Found: Cl = 34.92 per cent; Br = 26.24 per cent.

³ 1 cc. = 0.004192 gm. Br.

2,4,6-Trichlorophenoxyethyl dimethylamine Hydrochloride.—20 grams of 33 per cent aqueous dimethylamine were added to a solution of 15 grams of trichlorophenoxyethyl bromide in 50 cc. of benzene. The flask containing the mixture was stoppered and frequently shaken during the course of three days, the aqueous layer, which at first floated on the benzene, gradually sinking to the bottom. The mixture was finally warmed for one hour on the water bath and then evaporated *in vacuo* to a syrup. This was taken up in water, made alkaline with sodium hydroxide, and extracted with ether. The residue obtained by concentration of the ether layer was treated with concentrated hydrochloric acid, diluted with absolute alcohol, and precipitated with ether. 7 grams of the hydrochloride were obtained in this way in the form of long, silky needles, melting at 187–9°. It dissolves readily in water and absolute alcohol.

0.1988 gm. of substance required 6.5 cc. AgNO₃ Solution I.

Calculated for C₁₀H₁₁ONCl₃·HCl: Cl⁻ = 11.62 per cent.

Found: Cl⁻ = 11.51 per cent.

2,4,6-Trichlorophenoxyethyl piperidine Hydrochloride.—7 grams of piperidine were added to a solution of 10 grams of trichlorophenoxyethyl bromide in 25 cc. of benzene, after which the mixture was warmed on the water bath for two hours. The mixture was cooled, diluted with ether, and the piperidine hydrobromide filtered off and washed with ether. The filtrate was distilled to small bulk, made alkaline with sodium hydroxide, and freed from unchanged piperidine by distillation with steam, a small amount of the new base being also carried over. The residue was shaken out with ether, which was then dried and evaporated to small bulk. As the resulting oil did not crystallize, it was taken up in a little absolute alcohol, treated with an excess of concentrated hydrochloric acid, and evaporated on the water bath until crystallization commenced. The mixture was then cooled and treated with ether until the maximum amount of hydrochloride had been precipitated. Yield: 7 grams. The salt melts at 188–9° and is easily soluble in water and alcohol.

0.2036 gm. of substance required 5.95 cc. AgNO₃ Solution I.

Calculated for C₁₂H₁₆ONCl₃·HCl: Cl⁻ = 10.27 per cent.

Found: Cl⁻ = 10.28 per cent.

p-Bromophenoxyethyl Bromide.—Phenoxyethyl bromide was treated in chloroform solution with 1.1 mols. of bromine. The solution was allowed to stand several hours and was then concentrated to a syrup. When this was chilled it formed a crystalline mass which was rubbed up with ligroin and sucked off. Recrystallized first from ligroin, then from absolute alcohol, it forms rhombs which melt at 56–7° (corrected) with slight preliminary softening. The bromide dissolves readily in benzene and ether, difficultly in cold absolute alcohol.

0.1466 gm. of substance (Carius) gave 0.1970 gm. AgBr.

Calculated for $C_6H_5OBr_2$: Br = 57.11 per cent.

Found: Br = 57.19 per cent.

p-Bromophenoxyethylhexamethylenetetraminium Bromide.—This was obtained by boiling the components in dry chloroform. The rate of addition was very slow. The salt decomposes at 185–6°, is difficultly soluble in water, and gives a faint yellow color with sulphuric acid.

0.2707 gm. of substance required 6.57 cc. AgNO₃ Solution I.

Calculated for $C_{14}H_{20}ON_4Br_2$: Br = 19.03 per cent.

Found: Br = 19.25 per cent.

Tribromo-*p*-methylphenoxyethyl Bromide (Tribromo-*p*-cresoxyethyl Bromide).—17 grams of tribromo-*p*-cresol, a solution of 4 grams of 50 per cent aqueous sodium hydroxide in 25 cc. of absolute alcohol, and 75 grams of ethylene bromide were boiled for four hours. The product was distilled with steam and the residue treated with alkali to dissolve the unchanged cresol. The undissolved oil was taken up in chloroform, dried, and evaporated to small bulk. The residue crystallized on cooling. Recrystallized from acetic acid, in which it is difficultly soluble in the cold, it melts at 50–1° (corrected), with preliminary softening. It is easily soluble in benzene and acetone, less so in cold absolute alcohol.

0.1248 gm. of substance (Carius) gave 0.2090 gm. AgBr.

Calculated for $C_8H_8OBr_4$: Br = 70.77 per cent.

Found: Br = 71.30 per cent.

Tribromo-*p*-methylphenoxyethylhexamethylenetetraminium Bromide.—Equimolecular amounts of the components were allowed to

stand in chloroform solution for four months. The deposited salt was filtered off, dried, and washed first with a little ice water, then with alcohol and ether. It melts at 181° , is very difficultly soluble in water, and gives no color with sulphuric acid.

0.1965 gm. of substance required 3.4 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{12}\text{H}_{20}\text{ON}_4\text{Br}_4$: $\text{Br}^- = 13.50$ per cent.

Found: $\text{Br}^- = 13.73$ per cent.

Tribromo-p-methylphenoxyethylpiperidine Hydrobromide.—This salt was prepared for comparison with the hexamethylenetetraminium salt. 5 grams of tribromo-*p*-cresoxyethyl bromide were dissolved in 10 cc. of benzene and 2.5 grams of piperidine added. After boiling on the water bath for several hours, the precipitated piperidine hydrobromide was filtered off and washed with benzene. The filtrate was concentrated to a syrup, taken up in absolute alcohol, and treated with an excess of hydrobromic acid. The salt crystallized upon the addition of water, and was filtered off and washed with acetone and ether. The yield was almost quantitative. As the product had an irritating odor, it was recrystallized from a small amount of absolute alcohol. It is fairly readily soluble in water and is precipitated from its solution by hydrobromic acid. Heated in a tube sealed at both ends, it melts and decomposes at $223\text{--}30^{\circ}$.

0.2151 gm. of substance required 3.95 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{12}\text{H}_{18}\text{ONBr}_3 \cdot \text{HBr}$: $\text{Br}^- = 14.89$ per cent.

Found: $\text{Br}^- = 14.57$ per cent.

Tetrabromo-p-methylphenoxyethyl Bromide.—In one experiment on the preparation of the tribromo compound in which an impure tribromo-*p*-cresol, containing apparently some tetrabromo cresol, had been used as starting material, a small amount of the tetrabromo compound was separated from the reaction product by virtue of its greater insolubility in absolute alcohol. It formed long, delicate, glistening needles, which, when recrystallized twice from acetic acid, melted at $106\text{--}7.5^{\circ}$ with preliminary softening.

0.1632 gm. of substance (Carius) gave 0.2898 gm. AgBr .

Calculated for $\text{C}_8\text{H}_7\text{OBr}_4$: $\text{Br} = 75.19$ per cent.

Found: $\text{Br} = 75.57$ per cent.

o-Acetaminophenoxyethyl Bromide.—75 grams of *o*-acetaminophenol were dissolved in a mixture of 40 grams of 50 per cent aqueous sodium hydroxide and 250 cc. of absolute alcohol. After adding 500 grams of ethylene bromide, the mixture was boiled for two hours. The alcohol and excess of ethylene bromide were distilled off with steam, after which the insoluble material solidified on cooling. This was dried and taken up in chloroform, in which the ethylene ether (30 to 40 grams) was found to be insoluble. The filtrate was evaporated to small bulk, precipitated with ligroin, and the product recrystallized from dilute alcohol. Yield: 60 grams. Recrystallized twice from absolute alcohol, the bromide forms needles melting at $90-0.5^{\circ}$ (corrected) with preliminary softening. It is readily soluble in benzene and chloroform, less so in ether.

0.2769 gm. of substance (Kjeldahl) required 10.85 cc. $\frac{N}{10}$ HCl.

0.1806 gm. of substance gave 0.1330 gm. AgBr.

Calculated for $C_{10}H_{12}O_2NBr$: N = 5.43 per cent; Br = 30.96 per cent.

Found: N = 5.49 per cent; Br = 31.33 per cent.

o-Acetaminophenoxyethylhexamethylenetetraminium Bromide.—17 grams of the salt were obtained from a mixture of 13 grams of bromide, 7 grams of hexamethylenetetramine, and 70 cc. of chloroform allowed to stand at room temperature for five months. The salt was further purified by boiling with dry acetone, after which it melted at about 160° . It is slowly, but freely, soluble in water and gives a faint pinkish color with sulphuric acid.

0.2069 gm. of substance required 5.15 cc. $AgNO_3$ Solution I.

Calculated for $C_{18}H_{24}O_2N_4Br$: Br = 20.09 per cent.

Found: Br = 19.74 per cent.

o-Aminophenoxyethyl Bromide Hydrobromide.—*o*-Acetaminophenoxyethyl bromide was saponified by boiling for two hours with a mixture of one part of aqueous hydrobromic acid (d 1.49) and two parts of absolute alcohol. The amino hydrobromide, which separated in long needles on cooling, was recrystallized from absolute alcohol. It melts and decomposes at $193-8^{\circ}$. In aqueous solution it gives a lilac color with ferric chloride.

0.1997 gm. of substance gave 0.2543 gm. AgBr.

Calculated for $C_6H_7ONBr \cdot HBr$: Br = 53.83 per cent.

Found: Br = 54.20 per cent.

o-Aminophenoxyethyl bromide was obtained by adding just enough sodium hydroxide to a cold solution of the above salt. It separates as an oil which rapidly changes to glistening leaflets. These were quickly dried and recrystallized from ligroin, since, on keeping, the base liquefies owing to internal condensation. It melts at $36-7^{\circ}$ (corrected).

0.2392 gm. of substance (Kjeldahl) required 11.05 cc. $\frac{N}{10}$ HCl.

Calculated for $C_8H_{10}ONBr$: N = 6.48 per cent.

Found: N = 6.47 per cent.

o-Aminophenoxyethylpiperidine Hydrochloride.—15 grams of *o*-acetaminophenoxyethyl bromide in 100 cc. of benzene were treated with 12 grams of piperidine and warmed gently. The reaction proceeded smoothly, accompanied by the separation of piperidine hydrobromide. After standing over night, the mixture was heated for a short time on the water bath, cooled, treated with an equal volume of ether, and filtered. The filtrate was evaporated *in vacuo* to a syrup, taken up in absolute alcohol, and treated with an equal volume of concentrated hydrochloric acid. The mixture was boiled for two hours in order to remove the acetyl group, evaporated to small bulk *in vacuo*, taken up in absolute alcohol, and precipitated with ether. Yield: 15 grams. Recrystallized from absolute alcohol, the salt forms short, thick prisms melting at $184-5.5^{\circ}$. It dissolves readily in water and hot absolute alcohol, the former solution giving a deep brown color when warmed with a drop of ferric chloride solution.

0.2326 gm. of substance required 9.07 cc. $AgNO_3$ Solution I.

Calculated for $C_{12}H_{20}ON_2 \cdot HCl$: Cl = 13.82 per cent.

Found: Cl = 13.72 per cent.

o-Aminophenoxyethylpiperidine.—In D. R.-P. 88502⁴ it is stated that this substance is formed by the reduction of *o*-nitrophenoxyethylpiperidine, but no description of the product is given. As prepared by decomposition of the hydrochloride with sodium carbonate it forms a rapidly solidifying oil, which, recrystallized first from 50 per cent alcohol, then from ligroin, forms almost colorless diamond-shaped plates which melt at $69-70^{\circ}$ (corrected) with preliminary

⁴ Friedlaenders Fortschr. d. Teerfarbenfabrikation, iv, p. 814, 1894-97.

softening. These are readily soluble at room temperature in the neutral organic solvents, excepting ligroin.

0.1243 gm. of substance gave 13.95 cc. N (774 mm. and 24°).

Calculated for $C_{13}H_{20}ON_2$: N = 12.72 per cent.

Found: N = 12.76 per cent.

p-Acetaminophenoxyethylhexamethylenetetraminium Bromide.—13 grams of *p*-acetaminophenoxyethyl bromide, 7 grams of hexamethylenetetramine, and 70 cc. of chloroform were allowed to stand for five months at room temperature. 13 grams of the crude salt were obtained. After boiling out with dry acetone it melted at 196–8°. It is difficultly soluble in water and darkens in sulphuric acid, giving a faint blue-gray color.

0.1972 gm. of substance required 4.9 cc. $AgNO_3$ Solution I.

Calculated for $C_{16}H_{24}ON_2Br$: Br = 20.09 per cent.

Found: Br = 19.71 per cent.

o-Carbomethoxyphenoxyethyl Bromide (2-Bromoethoxybenzoic Acid Methyl Ester).—50 grams of methyl salicylate, a solution of 28 grams of 50 per cent aqueous sodium hydroxide in 200 cc. of absolute alcohol, and 200 grams of ethylene bromide were boiled for four hours. The mixture was diluted with water, made alkaline, and extracted with ether. The residue obtained from the ether extract was fractionated *in vacuo*. The bromoethyl ether boils at 186–8° at 20 mm., and, on standing, partly solidifies. The crystalline portion was recrystallized twice from absolute alcohol, forming large, thick, transparent plates which melt at 37.5–8.° (corrected). The compound is readily soluble at room temperature in the usual solvents except water. The yield was poor.

0.1741 gm. of substance (Carius) required 0.1239 gm. $AgBr$.

Calculated at $C_{10}H_{11}O_2Br$: Br = 30.85 per cent.

Found: Br = 30.30 per cent.

With hexamethylenetetramine in dry chloroform, the bromide gave only hexamethylenetetraminium bromide.

2-Bromoethoxybenzamide.—50 grams of salicylamide were dissolved in a solution of 8 grams of sodium in 250 cc. of absolute alcohol and 200 grams of ethylene bromide added. After boiling

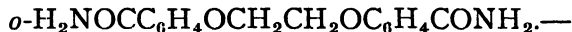
for one hour, the alcohol and excess of ethylene bromide were blown off with steam. The residual mixture was made alkaline and chilled, causing the gradual solidification of the oily reaction product. This was filtered off, boiled up with chloroform, allowed to cool, and the solution filtered from the residue of ethylene compound (14 grams; see below). The addition of ligroin to the filtrate resulted in the precipitation of the 2-bromoethoxybenzamide, which melted at 112–4°. Yield: 23 grams.

0.2449 gm. of substance (Kjeldahl) required 10.44 cc. $\frac{N}{16}$ HCl.

Calculated for $C_8H_{10}O_2NBr$: N = 5.74 per cent.

Found: N = 5.97 per cent.

Salicylamide Ethylene Ether,



A portion of the ethylene ether obtained above was recrystallized twice from acetic acid and washed with acetone. It forms crystals which melt at 211.5° (corrected) and are difficultly soluble at room temperature in the usual solvents.

0.1948 gm. of substance (Kjeldahl) required 13.25 cc. $\frac{N}{16}$ HCl.

Calculated for $C_{16}H_{18}O_4N_2$: N = 9.33 per cent.

Found: N = 9.53 per cent.

(2) Bromoethyl Esters and Their Hexamethylenetetraminium Salts.

Acetoxyethylhexamethylenetetraminium Bromide.—9 grams of bromoethyl acetate (ethylene bromoacetin) and 7 grams of hexamethylenetetramine in 50 cc. of dry chloroform were heated in a sealed tube for one hour at 105°. The salt separated as silky needles which dissolved readily in water and melted at 172–3°. The usual method of preparation resulted in the formation of an impure salt.

0.2058 gm. of substance required 6.95 cc. AgNO_3 Solution I.

Calculated for $C_{10}H_{18}O_2N_4Br$: Br = 26.03 per cent.

Found: Br = 26.79 per cent.

Benzoyloxyethylhexamethylenetetraminium Bromide.—12 grams of bromoethyl benzoate⁵ and 7 grams of hexamethylenetetramine

⁵ K. Auwers and E. Bergs: *Ann. d. Chem.*, cccxxxii, p. 209, 1904.

were boiled for two hours in 200 cc. of dry toluene. The salt formed a thick mass of glistening needles on cooling. It melts to a yellow paste at $164-5^{\circ}$ and is rather difficultly soluble in water.

0.2021 gm. of substance required 5.6 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{18}\text{H}_{21}\text{O}_4\text{N}_2\text{Br}$: Br = 21.66 per cent.

Found: Br = 21.98 per cent.

Bromoethyl p-Nitrobenzoate.—28 grams of ethylene bromohydrin and 40 grams of *p*-nitrobenzoyl chloride were heated on the water bath. Hydrochloric acid was slowly evolved, and the solution was finally heated in an oil bath at 140° until the evolution of gas ceased. The product, which solidified on cooling, was taken up in ether and shaken out with dilute alkali to remove *p*-nitrobenzoic acid. After concentration of the ether solution the ester solidified. Yield: 41 grams. Recrystallized twice from absolute alcohol, it forms faintly yellow rhombic crystals melting at $47-50^{\circ}$ (corrected). It is readily soluble in benzene, chloroform, and ether.

0.1990 gm. of substance (Kjeldahl) required 6.90 cc. $\frac{N}{10}$ HCl.

0.1814 gm. of substance (Carius) gave 0.1192 gm. AgBr.

Calculated for $\text{C}_9\text{H}_9\text{O}_4\text{NBr}$: N = 5.11 per cent; Br = 29.16 per cent.

Found: N = 4.86 per cent; Br = 27.97 per cent.

p-Nitrobenzoyloxyethylhexamethylenetetraminium Bromide.—10 grams of the bromoethyl ester and 5 grams of hexamethylenetetramine in 100 cc. of dry toluene were boiled for five hours. The liquid gradually became filled with a mass of glistening leaflets. 4 grams of the salt were obtained in this way, while the mother liquors yielded an additional quantity when boiled again. The compound melts with decomposition at $190-1^{\circ}$ and is difficultly soluble in water.

0.1890 gm. of substance required 4.65 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{18}\text{H}_{20}\text{O}_4\text{N}_2\text{Br}$: Br = 19.31 per cent.

Found: Br = 19.52 per cent.

p-Nitrobenzoyloxyethylhexamethylenetetraminium Iodide.—Before the above bromide had been prepared, an attempt had been made to combine chloroethyl *p*-nitrobenzoate with hexamethylenetetramine. As this had not been successful under the conditions tried, resort was

had to the iodoethyl ester. This reacted very slowly with the base in boiling chloroform, precipitation of the salt being incomplete even after several days. The compound forms glistening yellow platelets which melt at $176-8^{\circ}$ and are very difficultly soluble in cold water.

0.2017 gm. of substance required 4.45 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{15}\text{H}_{20}\text{O}_4\text{N}_2\text{I}$: I = 27.53 per cent.

Found: I = 27.78 per cent.

During the present investigation the following bromoethyl esters were prepared, but because of pressure of other work no attempt was made to obtain the hexamethylenetetraminium salts from them.

Bromoethyl Acetylsalicylate.—To a well chilled solution of 10 grams of ethylene bromohydrin in 45 grams of pyridine were slowly added 16 cc. of acetylsalicylic chloride.⁶ After several hours the mixture was poured into an excess of iced 25 per cent sulphuric acid. The red oil which separated was taken up in ether, washed with dilute sulphuric acid and water, dried over calcium chloride, and, after evaporation of the ether, fractionated in a high vacuum. The ester boils at $150-60^{\circ}$ at 0.7 mm. and crystallizes on cooling. Recrystallized twice from absolute alcohol, it forms thick plates and prisms possessing an aromatic odor. It melts at $62-2.5^{\circ}$ (corrected) with preliminary softening and dissolves readily in ether, chloroform, and benzene.

0.1575 gm. of substance (Carius) gave 0.1029 gm. AgBr .

Calculated for $\text{C}_{11}\text{H}_{11}\text{O}_4\text{Br}$: Br = 27.84 per cent.

Found: Br = 27.81 per cent.

Bromoethyl Acetyl-p-cresotinate.—The crude chloride prepared from 40 grams of acetyl-p-cresotinic acid⁷ was dissolved in dry, alcohol-free ether and slowly added to a well chilled solution of 25 grams of ethylene bromohydrin in 125 grams of pyridine. After one-half hour the mixture was poured into water and the resulting heavy oil taken up in ether. This was shaken out successively with dilute sulphuric acid and sodium carbonate solution and dried. The residue obtained after evaporation of the ether crystallized on chill-

⁶ R. Anschütz: *Ann. d. Chem.*, ccclxvii, p. 172, 1909.

⁷ R. Anschütz and J. Sieben: *Ann. d. Chem.*, ccclxvii, p. 245, 1909.

ing and scratching, after which it was disintegrated with a little ligroin and sucked off. 25 grams of the ester were obtained after recrystallization from ligroin. Recrystallized from toluene with the aid of a freezing mixture, it forms delicate, glistening needles which melt at $62.5-3.^{\circ}$ (corrected) with slight preliminary softening. It is difficultly soluble in ligroin, easily in benzene and toluene.

0.1402 gm. of substance gave 0.0858 gm. AgBr.

0.1179 gm. of substance gave 0.2072 gm. CO_2 and 0.0458 gm. H_2O .

Calculated for $\text{C}_{12}\text{H}_{13}\text{O}_4\text{Br}$: C = 47.83%; H = 4.35%; Br = 26.54%.

Found: C = 47.92%; H = 4.35%; Br = 26.05%.

Bromoethyl Anisate.—18 grams of ethylene bromohydrin and 24 grams of anisoyl chloride (from anisic acid and thionyl chloride) were warmed on the water bath. When the vigorous evolution of hydrochloric acid had ceased, water was added and the mixture shaken out with ether. This was washed with dilute sodium carbonate solution, dried, and evaporated to small bulk. The residue boiled at $189-91^{\circ}$ at 14 mm., and yielded 27 grams of the ester. At 0.5 mm. the boiling point was 140° (corrected).

0.1915 gm. of substance required 14.05 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{10}\text{H}_{11}\text{O}_3\text{Br}$: Br = 30.84 per cent.

Found: Br = 30.76 per cent.

Bromoethyl *m*-Chloroacetylaminomethylbenzoate,



15 grams of *m*-carboxychloroacetylbenzylamine (*m*-chloroacetylaminomethylbenzoic acid)⁸ were converted into the chloride.⁹ This was dissolved in chloroform and slowly added to a well chilled solution of 8 grams of ethylene bromohydrin in 50 grams of pyridine. After fifteen minutes the mixture was poured into an excess of iced 25 per cent sulphuric acid and shaken out with ether. When this was evaporated the residue crystallized. 20 grams of the ester were obtained after recrystallization from dilute alcohol. Recrystallized twice from benzene containing a little ligroin, then twice from abso-

⁸ A. Einhorn and T. Mauermayer: *Ann. d. Chem.*, cccxliii, p. 295, 1905.

⁹ See Paper II of this series, this *Journal*, xx, p. 693, 1915.

lute alcohol, using bone-black, it forms minute, pale yellow prisms which melt at $107-8.5^{\circ}$ (corrected).

0.2010 gm. of substance (Kjeldahl) required 6.25 cc. $\frac{N}{10}$ HCl.

0.0806 gm. of substance gave 0.0780 gm. AgCl + AgBr.

Calculated for $C_{12}H_{18}O_4NClBr$: N = 4.19%; Cl = 10.60%; Br = 23.89%.

Found: N = 4.36%; Cl = 10.36%; Br = 23.35%.

THE QUATERNARY SALTS OF HEXAMETHYLENE- TETRAMINE.

VII. ω -HALOGEN DERIVATIVES OF ALIPHATIC-AROMATIC KETONES AND THEIR HEXAMETHYLENETETRAMINIUM SALTS.

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(Received for publication, April 30, 1915.)

The preparation of quaternary salts by the addition of hexamethylenetetramine to halogen ketones has already been the subject of a study by Mannich and Hahn,¹ who used these substances as the starting material for the preparation of a few aminoketones according to the method of Delépine.² In our study of the quaternary salts of this base, we have had occasion to extend the work considerably in this direction. The results obtained will be the subject of the following.

Contrary to the facts observed in the use of the chloroacetyl derivatives of amino compounds, which, as a rule, reacted very smoothly with hexamethylenetetramine, the ω -chloroacetophenones were found to be unsuitable. This was due partly to the slowness of the reaction and partly to the decomposition which accompanied prolonged heating of the components. The best results were obtained by employing the bromo- or iodoketones. These reacted very readily with the base and the products were easily isolated. The salts obtained from the bromo compounds were preferred to those derived from the iodides owing to their greater solubility.

In the course of the work, a number of new halogen ketones were

¹ C. Mannich and F. H. Hahn: *Ber. d. deutsch. chem. Gesellsch.*, xliv, p. 1542, 1911.

² M. Delépine: *Bull. Soc. chim.*, series 3, xiii, p. 356, 1895; xvii, p. 293, 1897.

prepared, following with slight variations the methods of Kunckell and his coworkers.³

EXPERIMENTAL.

ω-Bromoacetophenoneoxime and Hexamethylenetetramine.—6.9 grams of *ω*-bromoacetophenoneoxime⁴ were added to a warm solution of 4.6 grams of hexamethylenetetramine in dry chloroform. The reaction product precipitated immediately as a transparent gum. This gradually crystallized on standing several days with occasional rubbing. The crude salt thus obtained was ground up in a mortar with dry chloroform, filtered off, and washed well with the solvent. Yield: 11.2 grams. It melts with decomposition at 139°.

0.2040 gm. of substance required 6.05 cc. AgNO₃ Solution I.⁵

Calculated for C₁₄H₂₀ON₄Br: Br = 22.57 per cent.

Found: Br = 23.53 per cent.

p-Methylphenacylhexamethylenetetraminium Bromide.—12 grams of *p*-methylphenacyl bromide (*p*-tolyl bromomethyl ketone⁶) were added to a solution of 8 grams of hexamethylenetetramine in dry chloroform. After the evolution of heat had ceased, the solution was boiled a few minutes, cooled, filtered from a slight precipitate, and treated with several volumes of dry acetone. The salt separated as slightly pinkish, minute prisms which melted at 148–9° to an orange-brown liquid. It dissolves readily in water and chloroform. Yield: 13.9 grams.

0.2270 gm. of substance required 6.41 cc. AgNO₃ Solution I.

Calculated for C₁₀H₂₂ON₄Br: Br = 22.63 per cent.

Found: Br = 22.40 per cent.

p-Tolyl Iodomethyl Ketone (p-Methylphenacyl Iodide).—10 grams of *p*-tolyl chloromethyl ketone⁶ were dissolved in a little dry acetone and treated with 70 cc. of a normal solution of sodium iodide in dry acetone. After standing several days in the dark at room

³ F. Kunckell: *Ber. d. deutsch. chem. Gesellsch.*, xxx, pp. 577, 1713, 1897; xxxi, p. 169, 1898; xxxiii, pp. 2641, 2644, 1900.

⁴ H. Korten and R. Scholl: *Ber. d. deutsch. chem. Gesellsch.*, xxxiv, p. 1907, 1901.

⁵ 1 cc. = 0.00352 gm. Cl; 0.00793 gm. Br; 0.01259 gm. I.

⁶ Kunckell: *Ber. d. deutsch. chem. Gesellsch.*, xxx, pp. 577, 1713, 1897.

temperature the mixture was poured into water, precipitating an oil which soon solidified. Recrystallized twice from 95 per cent alcohol, the iodide forms cream-colored needles which melt at $42-3^{\circ}$ (corrected) and are readily soluble in the usual organic solvents. It has an acrid, irritating odor.

0.1463 gm. of substance (Carius) gave 0.1328 gm. AgI.

Calculated for C_8H_5OI : I = 48.82 per cent.

Found: I = 49.07 per cent.

p-Methylphenacylhexamethylenetetraminium Iodide.—On adding the iodoketone to a cold solution of the base in dry chloroform heat was immediately evolved and the solution soon set to a solid cake. The salt separates practically quantitatively in the form of colorless micro-needles which melt at $157-8^{\circ}$ to a red-brown tar. It is very difficultly soluble in cold water.

0.2761 gm. of substance required 6.93 cc. $AgNO_3$ Solution I.

Calculated for $C_{15}H_{21}ON_4I$: I = 31.72 per cent.

Found: I = 31.60 per cent.

o-Xylyl Bromomethyl Ketone.—11 grams of *o*-xylene were mixed with 22 grams of bromoacetyl bromide and added in small amounts to a suspension of 21.5 grams of powdered aluminium chloride in dry carbon disulphide. After the first vigorous evolution of hydrobromic acid had subsided the mixture was warmed slightly for about an hour and finally poured into ice water. The lower layer was taken up with ether, washed successively with dilute hydrochloric acid, dilute sodium carbonate solution, and finally with water. It was then dried, evaporated to small bulk, and diluted with ligroin before crystallization had set in. This modification of Kunckell's procedure was used throughout in preparing the alkyl-substituted chloro- and bromomethyl ketones used in the experiments described in this paper. By cooling the ligroin solution in a freezing mixture 21.5 grams of the *o*-xylyl bromomethyl ketone were obtained. Recrystallized from ligroin it forms large, slightly brownish needles with an irritating odor. It melts at $62-2.5^{\circ}$ (corrected) and is somewhat less soluble in ligroin than in the other usual organic solvents. In sulphuric acid it gives a greenish yellow color, in alcoholic sodium hydroxide an orange color. It is probably iden-

tical with the product melting at $63-4^{\circ}$ obtained by Armstrong and Kipping⁷ by brominating *o*-xylyl methyl ketone, although these authors assign the bromine to a position on the aromatic nucleus.

0.1461 gm. of substance required 12.40 cc. AgNO₃ Solution II.⁸

Calculated for C₁₀H₁₁OBr: Br = 35.19 per cent.

Found: Br = 35.58 per cent.

o-Xylyl Bromomethyl Ketone and Hexamethylenetetramine.—

This was prepared in the same way as *p*-methylphenacylhexamethylenetetraminium bromide. The crude product was purified by boiling for three-quarters of an hour with dry acetone. The salt melts at $137-8^{\circ}$ to a red liquid.

0.1916 gm. of substance required 5.12 cc. AgNO₃ Solution I.

Calculated for C₁₀H₁₂ON₄Br: Br = 21.77 per cent.

Found: Br = 21.20 per cent.

m-Xylyl Bromomethyl Ketone.—This was obtained in the same way as the *ortho* isomer. The yield was 11 grams. Recrystallized from ligroin with the aid of a freezing mixture, it forms colorless micro-leaflets with an irritating odor. It melts at $42-3^{\circ}$ (corrected), is very soluble at room temperature in the usual organic solvents, and gives an olive-yellow color when dissolved in concentrated hydrochloric or sulphuric acid.

0.1428 gm. of substance gave 0.1188 gm. AgBr.

Calculated for C₁₀H₁₁OBr: Br = 35.19 per cent.

Found: Br = 35.41 per cent.

m-Xylyl Bromomethyl Ketone and Hexamethylenetetramine.—

The addition of acetone to the filtered chloroform reaction mixture resulted in the deposition of the pure salt in the form of rosettes of needles. It melts at $145-6^{\circ}$ to an orange-red liquid.

0.2141 gm. of substance required 5.77 cc. AgNO₃ Solution I.

Calculated for C₁₀H₁₂ON₄Br: Br = 21.77 per cent.

Found: Br = 21.38 per cent.

p-Ethylphenyl Bromomethyl Ketone (*p*-Ethylphenacyl Bromide).

—8.3 grams of ethylbenzene (b p $131-5^{\circ}$) were mixed with 16

⁷ H. E. Armstrong and F. S. Kipping: *Jour. Chem. Soc.*, lxiii, p. 86, 1893.

⁸ 1 cc. = 0.004192 gm. Br.

grams of bromoacetyl bromide and added in small amounts to a suspension of 16 grams of powdered aluminium chloride in dry carbon disulphide. The resulting mixture was worked up as in the other cases. Yield: 10.9 grams. The ketone forms glistening micro-platelets which melt at $33-4^{\circ}$ (corrected) with slight preliminary softening and are very soluble in the organic solvents. It has a sharp odor, and gives a pale straw color with sulphuric acid.

0.1875 gm. of substance (Carius) gave 0.1595 gm. AgBr.

Calculated for $C_{10}H_{11}OBr$: Br = 35.19 per cent.

Found: Br = 36.20 per cent.

p-Ethylphenacylhexamethylenetetraminium Bromide.—When acetone is added to the filtered chloroform reaction mixture the salt gradually deposits as minute, glistening prisms. It melts and decomposes at $146-7^{\circ}$.

0.1794 gm. of substance required 4.90 cc. AgNO₃ Solution I.

Calculated for $C_{18}H_{23}ON_4Br$: Br = 21.77 per cent.

Found: Br = 21.67 per cent.

Mesityl Bromomethyl Ketone (2,4,6-Trimethylphenacyl Bromide).—This substance, prepared as in the previous cases, forms glistening, colorless plates which melt at $55-6^{\circ}$ (corrected) and are less soluble in ligroin than in the other organic solvents.

0.1475 gm. of substance (Carius) gave 0.1146 gm. AgBr.

Calculated for $C_{11}H_{13}OBr$: Br = 33.15 per cent.

Found: Br = 33.05 per cent.

m-Nitrophenacylhexamethylenetetraminium Bromide.—Equimolecular proportions of ω -bromo-*m*-nitroacetophenone and hexamethylenetetramine reacted immediately in warm, dry chloroform with separation of the crystalline salt. The mixture was heated to boiling for a few moments, after which it was allowed to cool and the substance filtered off. The salt was ground up with dry chloroform, filtered, and washed with dry acetone. It melts with decomposition at 157° and is difficultly soluble in the cold in water and absolute alcohol.

0.2246 gm. of substance required 10.86 cc. AgNO₃ Solution II.

Calculated for $C_{14}H_{13}O_2N_4Br$: Br = 20.77 per cent.

Found: Br = 20.27 per cent.

p-Acetaminophenyl Bromomethyl Ketone (*p*-Acetaminophenacyl Bromide).—30 grams of powdered aluminium chloride were added in small portions to a mixture of 12 grams of acetanilid, 30 grams of bromoacetyl bromide, and 70 cc. of dry carbon disulphide. After the reaction was completed the upper layer was poured off and the viscous, orange-red lower layer poured into ice water. The precipitate was filtered off and washed successively with dilute hydrochloric acid, water, and 50 per cent alcohol. Yield: 15.5 grams, after recrystallization from dilute alcohol or dilute acetic acid. Recrystallized from amyl alcohol, the ketone forms minute, cream-colored prisms which melt, when rapidly heated, at $190-3^{\circ}$ with decomposition. It is difficultly soluble in the cold in the common solvents.

0.3286 gm. of substance (Kjeldahl) required 13.05 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{10}H_{10}O_2NBr$: N = 5.47 per cent.

Found: N = 5.56 per cent.

p-Acetaminophenacylhexamethylenetetraminium Bromide.—Equal weights of the components were boiled in acetone for three hours. The salt separated as cream-colored prisms. These were filtered from the hot solution and washed well with acetone and chloroform. Yield: 75 per cent of the theory. When heated, the salt darkens and melts with decomposition at 194° . It is difficultly soluble in water.

0.2662 gm. of substance required 6.65 cc. $AgNO_3$ Solution I.

Calculated for $C_{10}H_{20}O_2N_5Br$: Br = 20.17 per cent.

Found: Br = 19.82 per cent.

p-Aminophenacylhexamethylenetetraminium Chloride.—6.1 grams of hexamethylenetetramine and 7.4 grams of *p*-aminophenyl chloromethyl ketone⁹ (*p*-aminophenacyl chloride) were boiled for about two hours in dry toluene. The process was accompanied by partial decomposition, but the light yellow micro-crystalline salt that had separated was filtered off, boiled first with chloroform, then with toluene, and dried. Yield: 2.6 grams. The product as obtained did not dissolve completely in water.

0.1936 gm. of substance required 6.46 cc. $AgNO_3$ Solution I.

Calculated for $C_{10}H_{20}ON_5Cl$: Cl = 11.45 per cent.

Found: Cl = 11.75 per cent.

⁹ Kunckell: *Ber. d. deutsch. chem. Gesellsch.*, xxxiii, p. 2645, 1900.

3-Acetamino-4-tolyl Bromomethyl Ketone (3-Acetamino-4-methylphenacyl Bromide).—This substance was prepared from *o*-acettoluide in the same way as the *p*-acetaminophenacyl bromide. The yield was not quite as good. Recrystallized twice from acetic acid, then from acetone, the ketone forms pale drab-colored, felted needles melting at 167–70° with preliminary softening. The positions assigned to the substituting groups are the same as those proven for the corresponding chloroketone by Kunckell.¹⁰

0.2448 gm. of substance (Kjeldahl) required 9.25 cc. $\frac{N}{N}$ HCl.

Calculated for $C_{11}H_{12}O_2NBr$: N = 5.18 per cent.

Found: N = 5.29 per cent.

3-Acetamino-4-methylphenacylhexamethylenetetraminium Bromide.—On boiling the components in dry chloroform for one-half hour the salt was precipitated as a gum which finally settled to the bottom of the flask as a clear, brownish oil. This became crystalline after adding dry acetone and rubbing. The salt was filtered off, ground up, and washed well with dry acetone. It forms a pale brown, micro-crystalline powder which dissolves readily in water and decomposes at 150–5°.

0.1869 gm. of substance required 9.19 cc. $AgNO_3$ Solution II.

Calculated for $C_{11}H_{12}O_2N_6Br$: Br = 19.47 per cent.

Found: Br = 20.61 per cent.

3-Acetamino-4-tolyl ω -Iodoethyl Ketone (3-Acetamino-4-methyl-iodopropiophenone).—20 grams of powdered aluminium chloride were added in small portions to a mixture of 8.5 grams of *o*-acettoluide, 20 grams of β -iodopropionyl chloride,¹¹ and 50 cc. of dry carbon disulphide. At first clumps of an addition product separated. These gradually melted with the slow evolution of hydrochloric acid and the mixture separated into two layers. After standing for two days, the upper layer was discarded and the lower layer poured into ice water. The pale greenish yellow precipitate was washed with dilute hydrochloric acid, water, and a little 50 per cent alcohol, and recrystallized, with bone-blackening, from dilute alcohol. Yield: 12.8 grams. Recrystallized from toluene, it forms faintly greenish,

¹⁰ Kunckell: *Chem. Zentralbl.*, lxxxiii, pt. i, p. 136, 1912.

¹¹ See Paper III of this series, this *Journal*, xxi, p. 136, 1915.

felted needles which melt at $142-3^{\circ}$ (corrected) to a dark liquid. It is difficultly soluble in the cold in chloroform, absolute alcohol, and toluene.

0.3796 gm. of substance (Kjeldahl) required 11.65 cc. $\frac{N}{N}$ HCl.

Calculated for $C_{12}H_{14}O_2NI$: N = 4.23 per cent.

Found: N = 4.30 per cent.

In order to determine the positions of the substituting groups, 3 grams of the ketone were oxidized in acetone solution, with occasional cooling, with 5.5 grams of potassium permanganate. The filtrate from the manganese dioxide yielded, on acidification, a precipitate of the corresponding acid. 0.45 gram of this was obtained in the form of minute, glistening needles after recrystallization from acetic acid. It melts at $279-81^{\circ}$ (corrected) and is therefore probably identical with the 3-acetamino-4-methylbenzoic acid [m. $267-70^{\circ}$ (uncorrected)] obtained by Kunckell¹⁰ by oxidizing 3-acetamino-4-tolyl chloromethyl ketone. After hydrolysis with hydrochloric acid it contains a diazotizable amino group.

0.2019 gm. of substance gave 12.8 cc. N (760 mm. and 22.5°).

Calculated for $C_{10}H_{11}O_2N$: N = 7.25 per cent.

Found: N = 7.13 per cent.

3-Acetamino-4-tolyl ω -Iodoethyl Ketone and Hexamethylenetetramine.—Equimolecular amounts of the components were warmed slightly in dry chloroform and allowed to stand over night. The resulting thick, crystalline mass was disintegrated and boiled for about ten minutes to complete the reaction. The salt was filtered off and warmed for two hours with dry acetone. When heated, it turns yellow at about 150° and melts at $161-2^{\circ}$ with gas evolution. It is difficultly soluble in water.

0.2066 gm. of substance required 4.35 cc. $AgNO_3$ Solution I.

Calculated for $C_{18}H_{20}O_2N_2I$: I = 26.93 per cent.

Found: I = 26.52 per cent.

p-Methoxyphenacylhexamethylenetetraminium Bromide.—Instantaneous addition took place when *p*-anisyl bromomethyl ketone¹² was added to a solution of hexamethylenetetramine in warm, dry

¹² F. Kunckell and W. Scheven: *Ber. d. deutsch. chem. Gesellsch.*, xxxi, p. 172, 1898.

chloroform. After several hours about one-half volume of dry acetone was added and the salt filtered off and washed well with a mixture of two parts of dry chloroform to one part of dry acetone. The yield was practically quantitative. The salt forms faintly yellow micro-crystals which melt with decomposition at 172° . It is difficultly soluble in water and gives a bright yellow color with sulphuric acid.

0.2001 gm. of substance required 5.42 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{16}\text{H}_{21}\text{O}_2\text{N}_4\text{Br}$: Br = 21.65 per cent.

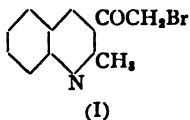
Found: Br = 21.49 per cent.

p-Ethoxyphenacylhexamethylenetetraminium Bromide.—*p*-Phenetyl bromomethyl ketone¹² reacted vigorously with hexamethylenetetramine in warm, dry chloroform. The resulting crystalline cake was disintegrated, ground up with dry chloroform, and boiled with this for one-half hour. After adding an equal volume of dry acetone and allowing to stand for several hours, the product was filtered off and washed with chloroform-acetone and finally dry acetone. The salt forms slightly yellow micro-prisms which melt with decomposition at $159-9.5^{\circ}$. It is difficultly soluble in water, somewhat soluble in chloroform, and gives with sulphuric acid a yellow color changing to orange-brown.

0.1973 gm. of substance required 5.18 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{16}\text{H}_{23}\text{O}_2\text{N}_4\text{Br}$: Br = 20.86 per cent.

Found: Br = 20.83 per cent.



β -[ω -bromoacetyl]-quinaldine (I).—25 grams of β -acetylquinaldine¹³ were dissolved in 100 cc. of concentrated hydrobromic acid (d 1.49), warmed to 50° and rapidly turbinated. To the solution were added, drop by drop, 25 grams of bromine dissolved in 50 cc. of hydrobromic acid.¹⁴ During the course of the reaction a yellow

¹³ J. Eliasberg and P. Friedländer: *Ber. d. deutsch. chem. Gesellsch.*, xxv, p. 1756, 1892.

¹⁴ According to the method of Kaufmann, *ibid.*, xlv, p. 1831, 1913.

hydrobromide of the bromoketone separated. After cooling, this was filtered off and washed with alcohol and ether. Yield: 35 grams. The free bromoketone was obtained from this by covering with water, in which it softens owing to dissociation, making alkaline with sodium carbonate, and shaking out with ether. The dried ethereal solution was evaporated to small bulk, leaving the bromoketone as an oil which solidified on chilling. Recrystallized first from ligroin, then toluene, then twice from methyl alcohol, it forms practically colorless rhombs which melt at $102-3^{\circ}$ (corrected) with slight preliminary softening. It is readily soluble in ether and chloroform and slowly decomposes when heated above 50° .

0.1380 gm. of substance gave 6.80 cc. N (764 mm. and 24.2°).

0.1510 gm. of substance gave 0.1081 gm. AgBr.

Calculated for $C_{12}H_{10}ONBr$: N = 5.30 per cent; Br = 30.26 per cent.

Found: N = 5.52 per cent; Br = 30.46 per cent.

β -[ω -bromoacetyl]-quinaldine and Hexamethylenetetramine.—Equimolecular amounts of the base and ketone (recrystallized once from ligroin) reacted almost instantaneously in dry, boiling chloroform, forming a solid, crystalline cake of the addition product. It melts with decomposition at about 170° . It is difficultly soluble in water, but easily so in 1 mol. of dilute hydrochloric acid.

0.2189 gm. of substance required 5.32 cc. $AgNO_3$ Solution I.

Calculated for $C_{18}H_{22}ON_4Br$: Br = 19.77 per cent.

Found: Br = 19.28 per cent.

THE QUATERNARY SALTS OF HEXAMETHYLENE- TETRAMINE.

VIII. MISCELLANEOUS SUBSTANCES CONTAINING ALIPHATICALLY BOUND HALOGEN AND THE HEXAMETHYLENETETRA- MINIUM SALTS DERIVED THEREFROM.

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In this paper we wish to present a number of substances which could not be logically incorporated with the previous papers. These are derivatives of a number of simple alkyl halides and halogenacyl esters. Several chloroacetyl amino compounds are also described.

EXPERIMENTAL.

(1) *Miscellaneous Alkyl Derivatives.*

Oxyethylhexamethylenetetraminium Iodide.—1 mol. of iodoethyl alcohol was added to a solution of 1 mol. of hexamethylenetetramine in dry chloroform. The mixture was filtered from a small amount of precipitate that separated almost immediately, and allowed to stand for two days in a warm place. The salt crystallized in the form of needles. It melts at 157° with decomposition and is very soluble in water.

0.1622 gm. of substance required 5.25 cc. AgNO₃ Solution I.¹

Calculated for C₈H₁₇ON₄I: I = 40.64 per cent.

Found: I = 40.74 per cent.

γ-Chloropropylhexamethylenetetraminium Bromide.—Trimethylene chlorobromide and hexamethylenetetramine reacted slowly in

¹ 1 cc. = 0.00352 gm. Cl; 0.00793 gm. Br; 0.01259 gm. I.

dry, boiling chloroform. Additional quantities of the quaternary salt may be obtained by continued boiling of the mother liquors. The salt forms irregular micro-prisms which are very soluble in water. When rapidly heated, it turns yellow above 140° , puffs up slightly, and finally melts with decomposition at $180-3^{\circ}$.

0.2456 gm. of substance required 8.23 cc. AgNO_3 Solution I.

Calculated for $\text{C}_6\text{H}_{18}\text{N}_4\text{ClBr}$: Br = 26.86 per cent.

Found: Br = 26.58 per cent.

γ -Oxypropylhexamethylenetetraminium Iodide.—This was prepared from 18.6 grams of trimethylene iodohydrin and 14 grams of hexamethylenetetramine in dry chloroform at room temperature. The portion (4 grams) that separated on standing over night was contaminated with hexamethylenetetraminium iodide, but the fraction (10 grams) separating during the next few days was free from this impurity.

0.2009 gm. of substance required 11.4 cc. AgNO_3 Solution II.²

Calculated for $\text{C}_6\text{H}_{18}\text{ON}_4\text{I}$: I = 38.92 per cent.

Found: I = 37.79 per cent.

Cetylhexamethylenetetraminium Iodide.—This was prepared in chloroform solution. The salt was recrystallized from absolute alcohol. It softens at 131° and melts gradually as the temperature is raised, liquefying completely and evolving gas at 157° . It is insoluble in cold water.

0.1940 gm. of substance required 7.58 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{22}\text{H}_{48}\text{N}_4\text{I}$: I = 25.77 per cent.

Found: I = 26.60 per cent.

Phenylethyl Iodide.—Phenylethyl chloride, prepared according to Barger,³ was treated with an excess of a normal solution of sodium iodide in dry acetone and boiled for four hours. The mixture, from which sodium chloride had separated, was poured into water and the precipitated oil taken up with ether. The ethereal solution was washed successively with dilute sodium bisulphite solution, water, sodium carbonate solution, and water. After drying over calcium

² 1 cc. = 0.00186 gm. Cl; 0.004192 gm. Br; 0.006657 gm. I.

³ G. Barger: *Jour. Chem. Soc.*, xcv, p. 2194, 1909.

chloride the ether was evaporated off. The residual oil was fractionated *in vacuo*, the portion boiling at 104.5–30.5° at 28 mm. being used directly for the preparation of the quaternary salt.

Phenylethylhexamethylenetetraminium Iodide.—The crude iodide reacted very slowly with hexamethylenetetramine in boiling chloroform. After several hours the mixture was diluted with more chloroform to dissolve unchanged base and filtered. An additional quantity was obtained by concentrating the filtrate and again boiling for several hours. The salt forms pale yellow cubes. When heated, it turns yellow and melts with decomposition at 159°.

0.2128 gm. of substance required 5.71 cc. AgNO₃ Solution I.

Calculated for C₁₄H₂₁N₄I: I = 34.11 per cent.

Found: I = 33.78 per cent.

The following substance was prepared since the publication of our first paper,⁴ in which the compound properly belongs:

o-Iodobenzylhexamethylenetetraminium Bromide.—An equimolecular amount of *o*-iodobenzyl bromide was added to a warm solution of hexamethylenetetramine in dry chloroform. The salt soon began to crystallize in the form of spherical aggregates of minute crystals. After several hours these were washed well with dry chloroform and dry acetone and were then pulverized and warmed with dry acetone in order to remove traces of tenaciously adhering iodobenzyl bromide. The salt is sparingly soluble in water, but dissolves more readily than the *p*-isomer. It effervesces and turns yellow at 173–5°.

0.1822 gm. of substance (Kjeldahl) required 16.65 cc. $\frac{N}{10}$ HCl.

0.2767 gm. of substance required 11.52 cc. AgNO₃ Solution II.

Calculated for C₁₃H₁₈N₄BrI: N = 12.82 per cent; Br = 18.29 per cent.

Found: N = 12.80 per cent; Br = 17.45 per cent.

(2) Esters of Monohalogen Acids.

Carbethoxyethylhexamethylenetetraminium Iodide.—Equimolecular quantities of β -iodopropionic acid ethyl ester and hexamethylenetetramine were boiled in dry chloroform. The salt separated as a yellowish oil. This was seeded with a few crystals obtained by

⁴ This *Journal*, xx, p. 659, 1915.

treating a portion with dry acetone and rubbing, causing solidification of the entire product. After continuing the boiling for one hour the precipitate of glistening leaflets was filtered off and washed thoroughly with dry chloroform. The salt dissolves readily in water and melts with decomposition at $137-8^{\circ}$.

0.2046 gm. of substance required 5.71 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{11}\text{H}_{21}\text{O}_2\text{N}_4\text{I}$: I = 34.48 per cent.

Found: I = 35.14 per cent.

sec.-Octyl Bromoacetate.—40 grams of bromoacetyl bromide were carefully added to 25 grams of *sec.*-octyl alcohol, with cooling. The mixture was then heated on the water bath until the evolution of hydrobromic acid ceased. The residue was fractionated *in vacuo*. The ester boils at 137° (corrected) at 18 mm. and possesses an odor resembling that of the alcohol.

0.1737 gm. of substance required 13.08 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{10}\text{H}_{19}\text{O}_2\text{Br}$: Br = 31.83 per cent.

Found: Br = 31.58 per cent.

With hexamethylenetetramine this product yielded a gelatinous salt which could not be crystallized.

Bornyl Bromoacetate and Hexamethylenetetramine.—Equimolecular amounts of the components were boiled in dry chloroform for one-half hour, filtered from the small amount of precipitated material, and treated with dry acetone. The solution gradually set to a thick mass of crystals of the salt. These were filtered off and washed with dry acetone. The compound sinters above 170° and melts at $178-81^{\circ}$. It is difficultly soluble in water.

0.2002 gm. of substance required 4.83 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{10}\text{H}_{17}\text{O}_2\text{N}_4\text{Br}$: Br = 19.24 per cent.

Found: Br = 19.14 per cent.

Menthyl Bromoacetate⁵ and Hexamethylenetetramine.—The salt was prepared in the same way as the bornyl compound, using dry ether as the precipitant instead of acetone. It melts at $153-4^{\circ}$ and is somewhat difficultly soluble in water.

0.2021 gm. of substance required 4.95 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{10}\text{H}_{17}\text{O}_2\text{N}_4\text{Br}$: Br = 19.15 per cent.

Found: Br = 19.43 per cent.

⁵ S. Smiles: *Jour. Chem. Soc.*, lxxxvii, p. 454, 1905.

Phenyl Bromoacetate and Hexamethylenetetramine.—Mannich and Drauzburg⁶ prepared the corresponding salts with phenyl chloroacetate and phenyl iodoacetate. As we found the former to be unsuitable for preparation on a large scale and as the latter proved to be insoluble in water, the bromide was prepared. The quaternary salt separates rapidly from a boiling solution of the components in dry chloroform. As precipitated, it contains chloroform of crystallization which was removed by boiling the product with dry acetone. It melts at 149–50° with decomposition, and is easily soluble in water.

0.2262 gm. of substance required 6.40 cc. AgNO₃ Solution I.

Calculated for C₁₁H₁₀O₂N₄Br: Br = 22.51 per cent.

Found: Br = 22.45 per cent.

*Tribromo-*p*-cresyl Bromoacetate.*—Tribromo-*p*-cresol was dissolved in normal sodium hydroxide solution and treated, drop by drop, with chilling and shaking, with 1.1 mols. of bromoacetyl bromide. The oil which remained after making the mixture alkaline crystallized on rubbing. Recrystallized twice from absolute alcohol, it forms cream-colored prisms which soften at 60° and melt at 60.5–1.5° (corrected). The ester is more difficultly soluble in alcohol, methyl alcohol, and ligroin than in the other organic solvents.

0.1394 gm. of substance (Carius) gave 0.2247 gm. AgBr.

Calculated for C₈H₅O₂Br₃: Br = 68.64 per cent.

Found: Br = 68.60 per cent.

The hexamethylenetetraminium salt of the ester was found to be insoluble in water and was not obtained in a state of purity.

o-Nitrophenyl Bromoacetate.—This substance was prepared in the same way as the preceding ester. Recrystallized twice from absolute alcohol, in which it is less soluble than in ether, benzene, and chloroform, it melts at 55.5–6.° (corrected) with slight preliminary softening.

0.2794 gm. of substance (Kjeldahl) required 11.30 cc. $\frac{N}{10}$ HCl.

0.1777 gm. of substance gave 0.1270 gm. AgBr.

Calculated for C₈H₅O₂NBr: N = 5.39 per cent; Br = 30.74 per cent.

Found: N = 5.67 per cent; Br = 30.41 per cent.

⁶ C. Mannich and W. Drauzburg: *Chem. Zentralbl.*, lxxxiii, pt. ii, p. 1817, 1912

o-Nitrophenyl Bromoacetate and Hexamethylenetetramine.—The salt separates practically quantitatively when a solution of the components in dry chloroform is boiled for twenty minutes. It forms slightly grayish micro-prisms which melt with decomposition at 149–50°. Its aqueous solution decomposes rapidly with separation of *o*-nitrophenol. Moist air also exerts a hydrolytic action. The corresponding chloride was prepared by Mannich and Drauzburg.⁶

0.1216 gm. of substance required 3.06 cc. AgNO₃ Solution I.

Calculated for C₁₄H₁₀O₄N₂Br: Br = 19.97 per cent.

Found: Br = 19.96 per cent.

Benzeneazo-β-naphthyl Chloroacetate.—4.2 cc. of chloroacetyl chloride were added to a solution of 12.4 grams of benzeneazo-β-naphthol in toluene. The mixture was boiled for nine hours and was then evaporated to small bulk. Separation of the ester was made more complete by the addition of ligroin. Yield: 6.7 grams. Recrystallized from absolute alcohol, it forms orange, felted needles which melt at 127–8° (corrected). It is very soluble in benzene, ether, and acetone, and dissolves in sulphuric acid with a purplish red color.

0.1310 gm. of substance gave 11.0 cc. N (750 mm. and 27.5°).

Calculated for C₁₈H₁₃O₂N₂Cl: N = 9.07 per cent.

Found: N = 8.63 per cent.

Oxyethyl Anisate (Oxyethyl p-Methoxybenzoate).—6.2 grams of ethylene glycol were dissolved in 30 grams of pyridine and chilled in a freezing mixture. After slowly adding a solution of 17 grams of anisoyl chloride in 15 cc. of dry chloroform, the mixture was allowed to stand at room temperature for one hour. It was then poured into iced 25 per cent sulphuric acid and shaken out with chloroform. The oil remaining after concentration was fractionated *in vacuo*. 10 grams of the oxyethyl anisate distilled at 161–2° at 0.75–0.85 mm. When redistilled it boiled at 149–50° (corrected) at 0.4 mm.

0.1147 gm. of substance gave 0.2569 gm. CO₂ and 0.0624 gm. H₂O.

Calculated for C₁₀H₁₂O₄: C = 61.19 per cent; H = 6.17 per cent.

Found: C = 61.11 per cent; H = 6.09 per cent.

Ethylene Anisate.—The undistilled residue obtained in the fractionation of the oxyethyl anisate solidified on cooling. Recrystallized

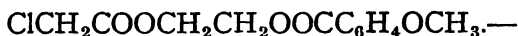
twice from acetic acid, it forms prisms which melt at $117-7.5^{\circ}$ with slight preliminary softening. It is difficultly soluble in cold absolute alcohol and ether, easily in benzene and chloroform.

0.1161 gm. of substance gave 0.2787 gm. CO_2 and 0.0547 gm. H_2O .

Calculated for $\text{C}_{18}\text{H}_{18}\text{O}_4$: C = 65.41 per cent; H = 5.49 per cent.

Found: C = 65.48 per cent; H = 5.27 per cent.

Chloroacetyloxyethyl Anisate,



A solution of 13.5 grams of crude oxyethyl anisate in 15 cc. of dry benzene was warmed with 7.5 grams of chloroacetyl chloride. After the evolution of hydrochloric acid had ceased the residue was fractionated *in vacuo*. The principal fraction boiled at $170-5^{\circ}$ at 0.5 mm. and solidified on standing. Recrystallized successively from dry ether and absolute alcohol with the aid of a freezing mixture, it melts at $45.5-6.5^{\circ}$ (corrected). It is very soluble in benzene and chloroform.

0.2822 gm. of substance required 19.39 cc. AgNO_3 Solution II.

Calculated for $\text{C}_{12}\text{H}_{13}\text{O}_5\text{Cl}$: Cl = 13.01 per cent.

Found: Cl = 12.75 per cent.

Chloroacetyloxyethyl Anisate and Hexamethylenetetramine.—

Equimolecular amounts of the components were boiled for one-half hour in dry chloroform, after which the solution was concentrated and filtered from a small amount of hexamethylenetetraminium chloride. The salt was then precipitated as a viscous oil by means of dry ether. The supernatant liquid was poured off and the oily salt dissolved in cold absolute alcohol. Ether was added until crystallization began, after which the mixture soon set to a solid mass. The salt was filtered off, washed, and placed in a desiccator as quickly as possible, owing to its hygroscopicity. When heated, it softens above 70° and gradually melts until it is completely fluid at 90° . It is easily soluble in water, alcohol, chloroform, and acetone.

0.1383 gm. of substance required 3.5 cc. AgNO_3 Solution I.

Calculated for $\text{C}_{18}\text{H}_{22}\text{O}_6\text{N}_4\text{Cl}$: Cl = 8.59 per cent.

Found: Cl = 8.91 per cent.

(3) Miscellaneous Amine and Hydrazine Derivatives.

ω-Chloroacetylaminophenone.—20 grams of *ω*-aminoacetophenone hydrochloride were dissolved in about 150 cc. of water and covered with about 100 cc. of toluene. After adding 11 cc. of chloroacetyl chloride, 150 cc. of two-normal potassium hydroxide were added in small amounts, with vigorous shaking and cooling. The chloroacetyl derivative soon separated and was filtered off, washed with water and toluene, and recrystallized, with bone-blackening, from dilute alcohol. Yield: 11.5 grams. It forms micaceous scales melting at 124–6.5° with preliminary softening. It is difficultly soluble in the cold in benzene, toluene, and ether, and dissolves very readily in chloroform.

0.2289 gm. of substance (Kjeldahl) required 11.15 cc. $\frac{N}{10}$ HCl.

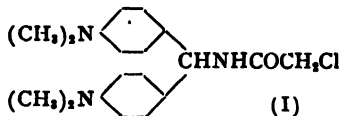
Calculated for $C_{10}H_{10}O_2NCl$: N = 6.62 per cent.

Found: N = 6.83 per cent.

ω-Chloroacetylaminophenone and Hexamethylenetetramine.—A chloroform solution of the components was boiled for one-half hour, evaporated to small volume, filtered from the slight precipitate, and treated with dry acetone. The salt separated as an oil which rapidly solidified, forming micro-platelets with a faint yellow-green tinge. It melts at 149–50° to an orange tar, is soluble in chloroform, and dissolves readily in water and alcohol.

Calculated for $C_{18}H_{22}O_2N_6Cl$: Cl = 10.08 per cent.

Found: Cl = 10.00 per cent.



Chloroacetylbis-[p-dimethylaminophenyl]-methylamine (*Chloroacetylleucoauramine*) (I).—8 grams of leucoauramine were dissolved in a mixture of benzene and toluene, 75 cc. of two-normal sodium hydroxide added, and then, drop by drop, 3 cc. of chloroacetyl chloride dissolved in toluene. Transitory colors developed, first green, then blue, then brown-violet, the color disappearing almost entirely in each case on shaking. Toward the end of the oper-

ation the chloroacetyl derivative started to crystallize out. To make this complete one-half volume of ligroin was added and the mixture allowed to stand in the ice box over night. The product was filtered off, washed well with water, and recrystallized from 95 per cent alcohol, forming almost colorless plates and needles which take on a greenish blue tinge on exposure to the air. Yield: 7 grams. The compound dissolves in acetic acid with a blue color, in sulphuric acid with a faint yellow color, and is readily soluble in chloroform and acetone, but insoluble in ether. It melts to a deep green liquid at $164-4.5^{\circ}$ (corrected) with slight gas evolution.

0.2442 gm. of substance (Kjeldahl) required 20.9 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{19}H_{24}ON_2Cl$: N = 12.16 per cent.

Found: N = 11.99 per cent.

Chloroacetylleucoauramine and Hexamethylenetetramine.—Equimolecular amounts of the components were boiled in dry chloroform solution for one and one-quarter hours. The olive-yellow solution was evaporated to small bulk and treated cautiously with dry acetone, causing the separation of the salt first as an oil. A portion of this was removed, rubbed up with acetone and ether, and crystallized by chilling. On seeding the main portion with this and slowly continuing the addition of dry acetone, the entire product was obtained in the crystalline state. After warming for one hour with dry acetone the salt still had a faint yellow color. It melts at 175° to a thick yellow liquid which decomposes at 179° . It is difficultly soluble in water but dissolves readily upon the addition of dilute acids.

0.3188 gm. of substance required 6.67 cc. $AgNO_3$ Solution I.

Calculated for $C_{20}H_{26}ON_2Cl$: Cl = 7.30 per cent.

Found: Cl = 7.36 per cent.

Chloroacetyltriphenylmethylaniline (Chloroacettriphenylmethylaniline).—11.7 grams of crude triphenylmethylaniline were obtained by passing dry ammonia into a mixture of 20 grams of triphenylmethyl chloride and 20 grams of naphthalene at 130° , taking up with ligroin, filtering off the ammonium chloride, saturating the filtrate with dry hydrochloric acid, suspending the resulting precipitate in dry benzene, passing in dry ammonia, again filtering, and evaporating the filtrate to dryness. The amine was taken up in benzene and

chloroacetylated as in the previous cases, completing the precipitation of the chloroacetyl derivative by means of ligroin. The compound was filtered off, washed with water, and recrystallized from acetic acid, forming snow-white, interlaced hairs. Yield: 5.9 grams. When recrystallized again from toluene and heated in a tube sealed at both ends it melts at $201-2.5^{\circ}$ (corrected). The substance dissolves readily in chloroform, less easily in benzene and hot acetic acid, and with difficulty in absolute alcohol. It gives a yellow color with sulphuric acid, deepening to a dark orange-brown on addition of a drop of aqueous potassium dichromate solution.

0.3072 gm. of substance (Kjeldahl) required 9.35 cc. $\frac{N}{10}$ HCl.

Calculated for $C_{21}H_{18}ONCl$: N = 4.17 per cent.

Found: N = 4.26 per cent.

Chloroacetyltriphenylmethylamine and Hexamethylenetetramine.

—After boiling for one hour the chloroform solution of the components was evaporated to small bulk and treated with several volumes of dry acetone. The precipitate of small, transparent cubes was boiled for one hour with dry acetone. When once isolated, the salt is difficultly soluble in chloroform. It also dissolves with difficulty in water and gives a yellow color with sulphuric acid. When heated it turns yellow at about 150° and melts with decomposition at $194-5^{\circ}$.

0.3173 gm. of substance required 6.27 cc. $AgNO_3$ Solution I.

Calculated for $C_{27}H_{30}ON_4Cl$: Cl = 7.46 per cent.

Found: Cl = 6.96 per cent.

β -Acetyl- α -chloroacetyl- α -phenylhydrazine and Hexamethylenetetramine.—The salt separated from the boiling chloroform solution of the components as long silky needles containing chloroform of crystallization. This was removed by boiling the compound for one-half hour with dry acetone. The salt melts at $192-3^{\circ}$.

0.1820 gm. of substance required 5.0 cc. $AgNO_3$ Solution I.

Calculated for $C_{18}H_{20}O_2N_4Cl$: Cl = 9.67 per cent.

Found: Cl = 9.67 per cent.

β -Chloroacetyl- α , α -phenylbenzylhydrazine.—20 grams of α , α -phenylbenzylhydrazine were chloroacetylated in toluene solution in

the same way as in the previous examples. As the chloroacetyl derivative did not separate from the toluene layer, the latter was shaken out first with dilute hydrochloric acid and then with dilute sodium carbonate solution. After the crystallization induced by the purification process had ended, the product was filtered off and combined later with an additional amount obtained by evaporating the filtrate to small bulk and adding ligroin. The substance was recrystallized by dissolving in hot benzene and adding an equal volume of ligroin. Yield: 17.8 grams. Recrystallized from methyl alcohol, it forms large, irregular crystals melting at $112.5-3.5^{\circ}$ (corrected). It is readily soluble in the usual organic solvents, except ligroin.

0.1537 gm. of substance gave 14.25 cc. N (755 mm. and 17°).

Calculated for $C_{18}H_{15}ON_2Cl$: N = 10.21 per cent.

Found: N = 10.64 per cent.

β -Chloroacetyl- α , α -phenylbenzylhydrazine and Hexamethylenetetramine.—After the solution of the components in dry chloroform had been boiled for eight hours it was concentrated and treated with several volumes of dry acetone. The salt slowly crystallized out in almost quantitative yield. It melts at $145-6^{\circ}$ to an orange liquid. It is readily soluble in water and dissolves in sulphuric acid with a brownish color.

0.2592 gm. of substance required 6.36 cc. $AgNO_3$ Solution I.

Calculated for $C_{21}H_{27}ON_6Cl$: Cl = 8.55 per cent.

Found: Cl = 8.64 per cent.

THE FORM OF THE EPITHELIAL CELLS IN CULTURES OF FROG SKIN, AND ITS RELATION TO THE CONSISTENCY OF THE MEDIUM.

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PLATES 6 TO 21.

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INTRODUCTION.

In a previous publication (1) the author has shown that the polygonal epithelial cells of frog skin assume a fusiform or thread-like shape ("spindle cells" and "thread-like columnar cells") shortly after they have wandered out into the medium. In this form they closely resemble connective tissue cells.

Champy (2) found in the case of the explanted kidney that both connective tissue and epithelial cells gradually took on an indefinite character, and he observed the same phenomenon in the explanted parotis, submaxillaries, and thyroids. It is of especial importance to note that the indefinite tissue of these organs is absolutely identical, and that, for example, the tissue derived from the kidney cannot be distinguished from that from the parotis. He calls this phenomenon "dedifferentiation," and attributes it to loss of function. It seems to us, however, that but little is gained by this explanation, for we possess no information regarding the functions of these organs in the embryonic stage and are unaware whether, for instance, the parotis or submaxillary functionates at all in the fetal organism. The point to be emphasized is rather the fact that organs as different as the kidney, salivary glands, and thyroids develop identical tissues as soon as the same chemicophysical conditions are brought to bear on the respective organs. This result signifies that the factors responsible for this equalization are pres-

ent in the culture medium. Thus, in view of the fact that we can modify at will the character of the medium, we are supplied with a definite means of further analyzing these phenomena.

It stands to reason that the above mentioned changes occurring in the cells are the result not of one factor, but of a number of different chemical and physical factors. In this paper, however, we intend to confine ourselves to the description of but one of these factors and to show in what way the consistency of the medium influences the form of the cell.

It is an easy matter to vary the firmness of the medium, which consisted of frog plasma and frog muscle extract obtained from the same species (*Rana pipiens*) that supplied the fragments of skin. The plasma of all individuals of the same species does not coagulate uniformly well, and it was soon apparent that this fact greatly influences the form which is subsequently assumed by the cells. Furthermore, by the addition of chicken plasma the frog medium can usually be made to gain a semi-firm consistency, and by the combined addition of chicken plasma and embryonic chicken extract the medium becomes very firm.

In previous experiments (1) we carried out prolonged observations on cultures that were cultivated in a pure frog medium (frog plasma + frog muscle extract), and found that the rim of tissue which formed around the explanted fragment of skin began by assuming the form of a compact membrane consisting of polygonal cells. Next the cells situated at the edge of the membrane began to project out into the medium, at the same time taking on an elongated spindle-shaped form (spindle cells). Many of these cells became detached from the membrane and wandered out into the plasma. By this means the edge of the membrane assumed a ragged appearance. Finally spaces occurred in the membrane which were bridged over by very elongated cells, often thread-like in appearance (thread-like columnar cells). As a result of successive processes the entire membrane gradually became divided up into long cells of the connective tissue type which strayed out into the medium.

It has now been shown that this change in the form of the tissue membrane and of its cells results from the gradual softening of the medium, and we are thus able, by the employment of suitable media, to produce artificially the individual stages of this process, accompanied by the characteristic form of the cells. The shape of the cells can be maintained permanently polygonal or fusiform, according as the medium selected is firm or soft.

As in our previous experiments, the material chosen was obtained from the dorsal skin of the adult leopard frog (*Rana pipiens*), which was cultivated in small fragments (approximately two square millimeters). The following are the results obtained with 179 cultures, and they are in accordance with the findings and records of the 480 cultures of our previous work.

EXPERIMENTAL.

Experiments with a Firm Medium.—We shall first describe the experiments made with a firm medium. A medium was considered firm when, in the process of changing, the culture could be cut out of the old medium enclosed in a firm block of medium. The firmness of these blocks was so great that they failed to bend over the edges of the cataract knife, even when they were considerably broader than the knife itself. We usually obtained such a medium by adding to two drops of frog plasma (F.Pl.)¹ one drop of frog muscle extract (F.E.), one drop of chicken plasma (Ch.Pl.), and one drop of embryonic chicken extract (Ch.E.).

Fig. 1 shows a culture (B 174) in the firm medium (F.Pl. + F.E. + Ch.Pl. + Ch.E.); photographed about twenty hours after explantation. A considerable rim of tissue has formed around the old fragment. It consists of strictly polygonal cells, and its line of demarcation towards the medium is uniform or only slightly irregular. The cells are closely pressed together. A vertical section of these cultures shows that the rim of tissue usually consists of from three to four layers of cells at the center, and of two layers at the periphery, and that the cells are polyhedral in shape, as shown in Fig. 2. In Fig. 3 are seen from above some of these polyhedral cells taken from a culture fixed *in toto* and stained with Giemsa (B 169); it was prepared in the same medium as culture B 174 in Fig. 1. Thus viewed we clearly see the polyhedral shape of the cells.

Fig. 4 (Culture B 114) represents a stained culture after a six days' sojourn in a firm medium. The culture was prepared on Dec. 15 and the following day showed but a relatively small rim of cells. On Dec. 17 the fragment of skin, together with the cell membrane, was placed in a fresh medium. On Dec. 19 the rim of cells had increased in size while remaining compact. It is noteworthy that no migration of cells had taken place. On the same day the old medium, which had remained completely firm, was exchanged for a fresh medium, into which was transferred the entire membrane of tissue. On the morning of Dec. 31 the medium showed signs of liquefying on one side. One protuberance into the medium had already formed, as well as a small cavity, which latter, however, was subsequently destroyed by fixation (to the left of the figure). After six days the culture was fixed. The membrane consisted exclusively of polygonal cells (Fig. 4), with the exception of that portion where the medium had begun to liquefy. Fig. 5 represents a part of the epithelial rim of Culture B 114 mag-

¹ We shall abbreviate as follows: frog plasma, F.Pl.; frog muscle extract, F.E.; chicken plasma, Ch.Pl.; chicken embryonic extract, Ch.E.

nified 1,000 times. The polygonal shape of the cells is distinctly seen. It should be noted that the tissue membrane of this culture (as also of all others subjected to the same conditions) maintained its compact form for six days without becoming loosened up, and that even the outermost cells of the rim, shown in Fig. 5, remained polyhedral in shape.

Fig. 6 was taken from a living culture (B 103) ten days after explantation. The culture was started on Sept. 12, 1914, and at first showed but slight signs of growth. The epithelium did not attain any considerable dimensions until Dec. 13, at which time it was quite thick. On Dec. 14 the medium was changed for the third time, whereupon it was found to be of exceedingly firm consistency. Thus it was possible to transfer the large tissue membrane without injury into the new medium. On Dec. 15 the rim of tissue was not as compact as in similar cultures which had been maintained under the same conditions. When the medium was changed on the same day it was also found to be less firm than that of the other cultures. Nevertheless, it was possible to cut out a block sufficiently firm to permit of the transfer of the entire rim of cells into the new medium. The cells of the outermost edges, however, which were the result of the growth from Dec. 14 to 15, tended to become massed together. On the 16th additional increase in the size of the cell membrane could be seen and new cells had been added. No migration of cells had taken place, however, and the membrane was quite compact. On Dec. 17 the medium was again changed. On Dec. 19 enlargement of the membrane could again be seen in certain sections, but the form of the membrane remained uniform and was unindented. No cell migration had taken place. At this stage the culture was photographed (Fig. 6). To the left of the figure can plainly be seen the thin layers of cells which sprouted out on the day preceding that on which the photograph was taken. They are separated from the remaining tissue by a lighter band of tissue. No detached cells can be seen around the culture. The latter, therefore, maintained for ten days consecutively a compact rim of tissue, which grew almost continuously and failed to undergo loosening up. The cells remained permanently polygonal in shape.

As long as the medium remained firm the same conditions were observed in all the other cultures subjected to the above treatment.

Experiments with a Medium of Semi-Firm Consistency.—A medium of semi-firm consistency was supplied by the addition of chicken plasma to the frog plasma and frog muscle extract (2 drops of F.Pl. + 1 drop of F.E. + 1 drop of Ch.Pl.). With a medium of this consistency it is still possible to remove a clean-cut block from the drop of plasma, but these blocks are so soft that if they extend beyond the edge of the knife their sides double over and adhere to the under surface of the knife. In order, therefore, to transfer into a fresh medium large blocks of this consistency with the enclosed culture every precaution must be used to avert this doubling over.

Moreover, it should be stated that owing to the extreme fluctuations in the consistency of the above mixture (F.Pl. + F.E. + Ch.Pl.) the results here obtained were by no means as uniform as those secured with the mixture composed of F.Pl. + F.E. + Ch.Pl. + Ch.E. Sometimes, for instance, a very firm medium is produced, at other times a soft one, such as results from the use of a pure

frog medium. But it is easy in each case to ascertain the degree of firmness. Here, however, we shall mention only the behavior of the cultures kept in a semi-firm medium; for their behavior when in a firm or soft chicken plasma mixture (F.Pl. + F.E. + Ch.Pl.) is the same as when kept in a firm medium (F.Pl. + F.E. + Ch.Pl. + Ch.E.) or in a pure frog medium (F.Pl. + F.E.), respectively.

Fig. 7 represents a culture about twenty hours old (B 86), cultivated in a mixture of F.Pl. + F.E. + Ch.Pl. The rather broad rim of cells is seen to be very dissimilar from the tissue membranes obtained in a firm medium. The central zone of cells cannot be clearly made out in the photograph, owing to too intensive staining. Nearly all the peripheral cells have assumed a spindle-shaped form. At several places sharp projections of cells can be seen jutting out into the medium, and at their extremities the typical fusiform cells are beginning to stray out individually into the medium. The process of migration is seen to be specially active at the projection seen in the right-hand upper corner of the photograph, as a result of which a small space has been formed in the membrane. The rim of tissue has consequently assumed an irregular, serrated appearance, and the medium in the neighborhood of the culture is beginning to fill up with detached fusiform cells that have strayed out, as can be clearly discerned in the figure.

The above characteristics are seen still more plainly in a three day old culture (B 7), which is shown under higher magnification in Fig. 8. The edges of the membrane project into the medium on all sides in the form of sharp tongues, the cells of which are elongated examples of the spindle form. An enormous number of cells have wandered out into the medium, and consequent upon this migration and upon the advance of the border cells, it is seen that behind the edge spaces of varying size are being formed in the membrane. These spaces, however, contain no liquid.

Figs. 9 a and 9 b are drawings of living cultures made without a camera lucida. They represent the process involved in the projecting out of the cells at the edge of the membrane and their ultimate detachment and migration (page 274).

It is possible to carry on for a prolonged period of time observations with chicken plasma cultures in the same manner as previously described in connection with the cultures grown in a firm medium, and furthermore to transfer them practically uninjured into fresh media. Cultures kept in a plasma of semi-firm consistency maintain permanently the characteristics described above, in contradistinction to those maintained in a firm medium, in which the cell membrane is permanently compact and the form of the cells unvaryingly polygonal, while the latter usually refrain from wandering off into the medium. Culture B 78 was observed for a period of twenty-two days. Not until the sixth day had a fairly large rim of epithelium formed; later, however, it became greatly reduced in dimensions, owing in part to considerable cell migration, and in part to losses incidental to change of medium. After fourteen days, as a result of this, only a small rim of cells remained, and some of the peripheral cells had become spherical in form, owing to insufficient nourishment, a circumstance which is apt to occur in any medium. On the fifteenth day, however, the peripheral cells

recovered, and began to form a new thin membrane, the edges of which showed the usual sharp indentations, and consisted of greatly elongated cells. Two subsequent transfers again occasioned a reduction of the membrane, and this was followed by renewed active growth of the fusiform cells accompanied by the usual migration of cells. The same process was repeated after another change of medium, at which stage the culture was photographed. Fig. 10 represents Culture B 78 twenty-one days after explantation. The central parts of the membrane consist chiefly of polygonal cells; the edges, however, show sharp and multiform indentations, and their cells, even those remote from the edge, are spindle-shaped. Cell migration can likewise be clearly seen.

After twenty-two days Culture B 78 was fixed and stained with Giemsa, in which condition it appears in Fig. 11. The membrane of cells is seen to be even more loosened up than on the preceding day, and long tissue processes, consisting of elongated spindle-shaped cells, are seen to project into the medium. The migration of cells has assumed exceptionally large proportions; the detached cells are for the most part fusiform in shape, but some are round.

Fig. 12 shows individual spindle-shaped cells, drawn under a high magnification. In Fig. 12 a we see the transformation of typical polygonal cells of the membrane into fusiform cells. Figs. 12 b and c represent typical examples of the isolated fusiform cells.

Experiments with a Soft Medium.—The phenomena which we have observed in the medium of semi-firm consistency occupy a position midway between those occurring in the firm and in the soft media. We shall now consider the latter. A soft medium is obtained by employing a pure frog medium (F.Pl. + F.E.). It is impossible to cut out little square blocks of this medium; on the contrary, these blocks invariably assume a spherical form, irrespective of the shape in which they have been cut out, and they furthermore have a tendency to adhere to the knife. Soft media can be drawn out in threads (isolation of the fibrin); they contain much liquid which is accumulated in the minute vacuoles in the network formed by the fibrin. These media rapidly succumb to complete liquefaction, whereupon a number of particular phenomena take place, which we must consider in connection with the rest, as they usually occur coincidentally.

Fig. 13 was photographed from a living culture (B 119), about twenty hours after explantation of the fragment. We are unable to determine whether in this culture a "secondary epithelial rim," as we termed in our first communication the compact rim of epithelium, preceded the loosening up stage, illustrated in this figure, as the former process may have developed over night. Nevertheless, in cultures consisting exclusively of frog medium the cells frequently begin to leave the fragment immediately in the form of a star-shaped membrane, with very ill defined edges; for these pure frog media usually become soft very soon, sometimes from the very outset. Fig. 13 gives a good impression of the condition of the cell membrane in a soft medium. Almost the entire rim of epithelium is drawn out in the form of long chains of cells and bands of tissue. Even the compact parts of the tissue membrane consist exclusively of elongated cells. On all sides large spaces are seen in the tissue membrane, lined by highly delicate, thread-like cells. These spaces contain no liquid, but soft medium. The whole form of the rim of tissue clearly indicates that the tissue is in a condition

of active movement, as can also clearly be seen in living cultures under the microscope. The chief characteristic of this particular culture is the enormous migration of cells. The entire drop of plasma is filled with wandering cells, which are partly fusiform in shape, partly round. In this culture whole fragments of tissue strayed out into the plasma. We were able to certify that they moved in a soft, not liquid substrate, by shaking the culture, a test invariably applied by us; for fragments floating in a liquid move from side to side, which did not occur in this case.

Fig. 14 shows Culture B 121 (stained with Giemsa), six days old, which was kept under parallel observation with the above mentioned Culture B 119 (Fig. 13). The culture was started on Dec. 15, and on Dec. 16 already possessed a fairly extensive star-shaped membrane of tissue, which projected out into the medium in long tissue processes, as is the rule in a soft frog medium culture. On Dec. 17 the medium was renewed, and on Dec. 19 the membrane of cells had attained considerable proportions. It was characterized by extremely long projections of tissue, as well as by bridges of thread-like columnar cells, and exceptionally active cell migration. The medium was renewed the same day, and on Dec. 21, six days after explantation, the culture was fixed, stained, and photographed (Fig. 14). The same characteristics can be noted as in Fig. 13, but more strongly emphasized.

Fig. 15 is a four day old culture in a pure frog medium photographed while living (B 117). The medium has partly liquefied and the resulting liquid has collected first of all in the holes in the tissue membrane caused by the migration of the peripheral cells. In such cases we are unable to decide whether these holes are primarily formed through the wandering out of the cells, as occurs in non-liquid cultures, or whether the membrane becomes torn by the vacuoles of liquefaction which are in process of being formed. The vacuoles, which at first are small, soon increase in size, the bridges of cells which separate them at the same time becoming thinner and thinner until at length they break away. By this means one or more larger vacuoles are formed, which in turn force the remaining part of the tissue to give way at the sides. Finally the cells are left in the form of a narrow wreath of cells encircling the large vacuole. In Fig. 15 can be seen the delicate, thread-like columnar cells which connect the ring of cells with the old fragment. At one place in this culture a single fragment of compact, but very thin tissue membrane yet remains; here the plasma had remained very firm, so much so that it was possible to cut out a block containing the membrane and transfer it into a new medium.

At this point it may be well to consider the behavior of the uppermost layers of epithelial cells. In Culture B 117 the same have become disconnected and are seen to lie over the old fragment and over a part of the cell membrane in the form of a thin membrane (Fig. 15) which possesses sharply defined edges while its cells have remained inactive. This phenomenon will be discussed in detail in a subsequent communication.

Cultures which are preserved in a pure frog medium, or in soft or liquid media generally, can only in rare cases, favored by every precaution, be maintained for any length of time as actively growing tissue membranes; usually the cells migrate in such large numbers that in a short time nothing remains of the

original membrane of cells. But it occasionally happens that a portion of the epithelium, instead of migrating, grows around the connective tissue either partially or completely, thus retaining its epithelial character. This particular phenomenon, however, which we have mentioned elsewhere (1), does not concern us here, and will be reserved for future consideration. It behooves us, rather, to follow the fate of certain cultures which when cultivated in a soft frog medium retained their membrane in a growing condition for a considerable length of time.

Culture B 77 was started on Nov. 28, and the tissue membrane took a fairly long time to develop. The records fail to show clearly at what time this took place. On Dec. 13, fifteen days after explantation, the membrane of cells still showed signs of growth at its edge, and was transferred on Dec. 14 to the new medium in an incomplete condition. On the following day, however, there were signs of renewed growth. On the same day the culture was again supplied with medium, and on Dec. 16 fresh growth of spindle-shaped cells was noted. At this stage the culture was photographed (Fig. 16), eighteen days after explantation. The cell membrane is seen to be exceedingly delicate and much thinner than that of a twenty-one day old culture grown in a medium of semi-firm consistency (Fig. 10). The tissue protuberances are much longer and narrower, and some of the cells are as fine as threads. The migration of cells is very active.

Similar conditions obtained in the case of Culture B 81, which was likewise started on Nov. 28 in pure frog medium. By Dec. 2 a narrow rim of epithelium with long protuberances and columns of thread-like cells had formed. On Dec. 13, one day after the medium had been changed for the eighth time, long fusiform cells still projected from the rim of membrane, and on Dec. 14 the membrane had again greatly increased in size. On Dec. 16, after two more changes of medium, the rim of tissue was still growing and projecting long processes into the medium. By Dec. 19 the entire membrane had become loosened up and an enormous number of bipolar fusiform and round cells could be seen in the plasma which had partially liquefied. The cells had strayed to the very limits of the drop of plasma. Fig. 17 shows the living culture photographed on the same day (twenty-one days after explantation). The spherical zone of liquefaction can be plainly seen, containing innumerable fusiform and round cells.

The behavior described above of the membranes of cells in connection with the old tissue fragment, is likewise characteristic of individual, isolated fragments of membrane when grown in daughter cultures. Fig. 18 shows an isolated fragment of membrane (Culture B 100 a) in a firm medium (F.Pl. + F.E. + Ch.Pl. + Ch.E.), and Fig. 19 represents a fragment of a tissue membrane (Culture B 119 a) in a soft medium (F.Pl. + F.E.). The former was taken from Culture 100, the latter from Culture 119. Culture B 100 a produced two days after isolation a fairly thick membrane, consisting of polygonal cells, whereas in Culture B 119 a twenty hours after isolation the elongated fusiform cells had formed a connective tissue-like network of cells. The thread-like columnar cells had developed exceptionally well in this culture, and can be seen in great numbers in Fig. 19.

At this stage it might be asked whether the above mentioned variations which we have ascribed to differences in the consistency of the medium, should not rather be attributed to the chemical differences prevailing in the three kinds of medium, in view of the fact that a firm consistency was secured by the addition to the pure frog medium of chicken plasma and chicken embryonic extract, which thus at the same time enriched the culture by the addition of the specific chemical materials characteristic of the body juices of a foreign species.

The extended period of growth of the said cultures is a sufficient indication that these foreign substances exert no injurious effect on them. For reasons not yet known it occasionally occurs that in spite of the addition of these foreign substances, the medium yet fails to solidify but remains soft, and in such cases the form of the membrane and cells is seen to be identical with those observed in a pure frog medium. On the other hand, it occasionally happens that a pure frog medium or one to which chicken plasma alone has been added, remains unusually firm; the result in such cases will be the formation of great compact rims of tissue and polygonal cells, such as are usually seen only in a culture composed of F.Pl. + F.E. + Ch.Pl. + Ch.E. The following illustrations prove this point. Fig. 20 shows a culture which was kept for about twenty hours in a pure frog medium. The rim of tissue is uncommonly thick and consists chiefly of polygonal cells; only in the left-hand top corner have the cells begun to assume a spindle form. Fig. 21 is a two day old culture (B 13), which was kept in a mixture of F.Pl. + F.E. + Ch.Pl. In this case the medium was exceptionally firm, the epithelium as a result thereof exceedingly compact, and the cells polygonal in shape. Fig. 22, on the other hand, shows culture B 100 which was grown in a mixture of F.Pl. + F.E. + Ch.Pl. + Ch.E., and which for a period of ten days possessed an exceedingly compact rim of tissue, consisting of polygonal cells. Here cell migration failed to occur. On the tenth day the culture was transferred into a new medium, which in spite of being composed of the customary mixture failed to coagulate entirely. The membrane thereupon immediately assumed the form characteristic of one grown in a semi-firm medium; *viz.*, in a combination of F.Pl. + F.E. + Ch.Pl. At the same time the cells became fusiform in shape and began to migrate.

Consistency of the Medium and Form of the Cells.

We have shown that the shape of the cells is dependent upon the consistency of the surrounding medium. We have also shown that the fusiform and thread-like epithelial cells of the connective tissue type, described in a former publication (1), arise only in media of a semi-firm or soft consistency, whereas epithelial cells grown in a firm medium remain permanently polyhedral. These observations

may be considered as a distinct advance, as they furnish information regarding the causes governing the shape of the cells. Nevertheless, we can not yet arrive at a complete understanding of this important morphological phenomenon, for we are confronted by the question as to how and why the shape of the cells is affected by change in the consistency of the medium. Neither our own experiments nor those of others at present offer a satisfactory explanation; but it is important definitely to realize along what lines among the knowledge and facts at our disposal we should search for a solution of the problem.

Our point of departure is a two-fold phenomenon: first the increased cell migration in a soft medium; and second the difference in the shape of the cells consequent upon the varying firmness of the medium.

Burrows was the first to consider in an exhaustive manner the effect of the dilution of plasma on the growth of tissue. He found that the cells move more rapidly and actively in plasma which has been diluted with tissue juices than in undiluted plasma, and he attributes this fact chiefly to the reduction in the fibrin content of the medium, consequent upon this dilution (3), and secondly, to the reduced density of the medium (4). From his demonstrations, however, we fail to learn that by diluting the plasma the fibrin content was diminished, for, according to Burrows, upon pressing out the liquid portion of the medium a firm block of fibrin again remained, which although reduced in thickness had nevertheless preserved its consistency.

Be that as it may, the problem of a reduction in the content of fibrin is not involved in our method, for in the media which contained relatively the largest quantity of fibrin (F.Pl. + F.E. + Ch.Pl.) the migration of cells was considerable, whereas in the media which contained less fibrin but more muscle extract (F.Pl. + F.E. + Ch.Pl. + Ch.E.) no migration whatever took place. As a matter of fact the relation between the cell migration and fibrin content was exactly the reverse from what would have been expected according to Burrows. With respect to density, however, our findings agree with those of Burrows. The maximum density coincides with a complete lack of isolated migrating cells, whereas in a pure frog medium, *i. e.*, one possessing a minimum density, the cells become detached from the tissue membrane and stray out into the medium in enormous quantities.

The form of the cells is closely connected with their movement; both phenomena originate from the same cause. By careful investigation of the above facts it will remain evident that neither can be ascribed to "activity" on the part of the cells, but are purely passive phenomena. The cell is impelled to move, and it receives its form by the same processes which necessitate movement, for which reason these two phenomena must be studied in combination.

We shall consider the various forms of cells in succession, beginning with the fusiform cells at the phase of their evolution from the polygonal type. This can be done without difficulty by selecting specimens whose medium is undergoing a change from the firm to the soft stage. Here we at once remark that the spindle-shaped cells are invariably formed from the border cells which are surrounded on three sides by the soft medium; they never arise from the polyhedral cells situated in the more central parts of the membrane where the latter is three to four layers thick. The border cells, as well as all the other cells constituting the membrane, possess a clearly differentiated ecto- and endoplasm. The latter is relatively dense and foamy in structure, containing vacuoles, granulations, and the nucleus. The ectoplasm, on the other hand, is homogeneous, and only plainly visible when the light in the microscope is partly shut off (Figs. 9 and 9 a). The above facts can be clearly distinguished in preparations *in toto*, which have been fixed with formol and stained with Giemsa (Fig. 12). The transition from the polygonal to the fusiform shape of the cells is a gradual process. First of all the ectoplasm projects from the edge of the membrane into the medium in the form of one or more exceedingly delicate but broad processes. This results at first in the cell's assuming a flask-shaped form (Fig. 9 a Ia), and moving out into the medium. Many of the cells, however, do not become immediately disconnected upon starting this movement, but remain attached on one side to several of the back-lying cells of the membrane, which often results in the posterior end of the projecting cell becoming long drawn out (Fig. 12 a, Fig. 9 b Ia). As soon as this connection is severed the posterior side of the cell usually contracts immediately, sometimes also the anterior end (Fig. 9 a III, 9 b IIa), after which the ectoplasm gradually disperses into the medium again from the anterior end of the cell, the posterior end again becoming drawn out into a long projection (Fig. 9 a IV and Fig. 9 b III a). By means of these processes uni-, bi-, and tripolar fusiform cells are formed. This migration of the peripheral cells causes the cells situated nearer the center of the membrane to be brought into contact with the medium, whereupon they undergo the same changes as the cells which first migrated. From the circumstance that the migrating cells fail to become dissociated immediately from the remaining cells of the membrane the latter similarly become drawn out, and thus chains or triangular processes are formed composed of elongated cells.

If we base our conclusions on the fact that the dispersion of the peripheral cells, which initiates the change in form, as well as the

movements of the polyhedral cells, occurs only in comparatively soft, but never in firm media, the fact is thus primarily established that the consistency of the medium is highly instrumental in causing these phenomena. Thus, there must exist a definite relation between the consistency of the medium and the consistency of the cytoplasm in order that the movement of the cells may be initiated. Additional light is thrown on this problem by a consideration of the observations which led Burrows to the formulation of his well thought out theory of cellular movement; for our own experiments fail to account for the dissociation from the tissue of the first migrating cells and the subsequent temporary contraction of their posterior end.

Burrows has proved that the mechanical laws underlying the movements of the cell are identical with Jacques Loeb's tropisms. According to Burrows, cells which are surrounded by a medium containing a uniform distribution of metabolic products are invariably spherical in shape in consequence of uniform contraction of the cytoplasm on all sides of the periphery of the cell. A culture, however, which has been freshly supplied with medium, does not possess this uniform distribution of metabolic products, for these are found in greatest concentration in the proximity of the original fragment, and in least concentration near the edge of the drop of medium. Thus, only the posterior end of the cell, *vis.*, that adjoining the old fragment of tissue, will become contracted. By means of repeated contraction of the posterior end and flowing out of the anterior end the cell is induced to move away from the old fragment.

This brilliant theory which is based on a series of painstaking observations lacks, however, one point; it fails to explain the extension of the anterior end of the cell. Our observations, therefore, here serve as a supplement to those of Burrows and are as follows: The cell plasma in flowing out simply obeys the law of gravity, and this movement is initiated at the moment when the relation between the consistency of the medium and the consistency of the cell plasma has attained a certain value, not yet ascertained, brought about by the reduction of the firmness of the medium.

This explanation likewise accounts for the shape of the fusiform cells; that is, of the unipolar ones. But even the bipolar form assumed by the migrating cells might be explained thus. We have seen that the peripheral cells, the free ends of which flow out into the medium, remain connected with the back-lying cells for some time longer, causing a passive elongation of the posterior end to take

place. Later this connection is severed and the posterior end of the cell again becomes first contracted and then elongated. It may safely be assumed that the part that was in the first instance played by one of the cells of the inner rows is now assumed by a fibrin-thread or by other fragments of tissue lying in the medium, to which the posterior end of the cell adheres. Thus, contraction can only take place at the moment that the tension, which is threatening the rupture of the progressively thinner posterior end, has attained a certain maximum, and is added to the contracting force resulting from the products of metabolism. The cell will, therefore, be compelled to continue its advance, assuming at times a bipolar and at times a unipolar form.

From the above it is primarily evident that an isolated cell must be invariably accompanied by the occurrence of the fusiform shape, unless the conditions become changed in such a way as to cause the cell to become round instead (page 277). As the isolation of cells is possible only in relatively soft media (in accordance with the law of gravitation), we are unable to find examples of fusiform cells occurring in firm media. It is easy to account for the polyhedral form of the cells in a firm medium which are united to form a membrane and which, as in the case of the flattened form assumed by the cells attached to a firm base, must be a result of surface tension. If in addition the cells are made to adhere to a firm base they become flat instead of polygonal in shape. For this reason the cells situated over the connective tissue of the explanted fragment are always flat, whereas the cells which are distributed on all sides in the form of a compact membrane and are uniformly surrounded by the firm medium, take on a polygonal shape, even if they were originally flat, as happens in the case of the cells of the middle epithelial layers. The cell retains this polyhedral shape until the firmness of the medium is reduced to the point at which it is possible for the border cells to flow out.

Thus we see that the cells' adoption of the polyhedral and fusiform shapes is not an active process, and not confined to a definite class of cells; for the epithelial cells can also become passively fusiform, in which condition it is hard to distinguish them from connective tissue cells. There is no doubt that the thread-like columnar

cells are likewise passively transformed epithelial cells; this change can at any time be observed in living cultures. They originate through tension from the polyhedral cells and are always seen to occur at places where the membrane has become torn and holes are formed in the tissue membrane. This, however, only occurs in semi-firm and soft media. In both media the peripheral cells wander more rapidly than the other cells, because a greater portion of their surface is in contact with the medium. By this means continuous tension is exerted on the central parts of the membrane, and the outer parts are thus gradually disconnected from the inner. The cells which are slow in giving up their connection become exceedingly drawn out before breaking off, as occurs in the case of the posterior end of the fusiform cells, and they thus bridge over the holes which have been formed. To this must be added in soft media the formation of vacuoles, by which the same result is effected.

We must now endeavor to account for the form of the round cells, of which mention has repeatedly been made. Round cells are found in all the media employed. The liquid medium contains them to the exclusion of all other forms (Fig. 17) whereas they are but rarely found in a firm medium and only under special conditions. In media which have liquefied but a few hours after the preparation of the culture, membrane formation never occurs, but all the cells rapidly become converted to the round type, and the entire rim of cells, even if when originally cultivated in a firm medium it consisted of a membrane of tissue, is then seen to be composed of loose round cells, irrespective of the frequency of the change of medium. In many cases the fusiform cells, after wandering about for a long time in an isolated state, become surrounded with a zone of liquid and then likewise become round in shape.

The round cells (Fig. 23) are distinguished from the other varieties by the fact that their cytoplasm almost always assumes a purple color in cultures stained with Giemsa, which suggests a difference in the chemical constitution of the round cell. Many of them possess a normal nucleus, some a pycnotic one (Fig. 23 a), and others no nucleus at all. Even cultures which have undergone complete loosening up and are surrounded by a fluid medium are still seen to contain a considerable number of normal round cells. Thus, the round

cell seen in Fig. 23 c originated in a culture (B 81) which had undergone complete loosening up (Fig. 17).

It is seen that the round cells are yet alive from the circumstance that many of them resume the fusiform shape as soon as they are placed in an appropriate medium. This was seen to occur in living cultures observed under the above conditions. Moreover, it is also possible in living cultures to witness the conversion of fusiform into round cells. Fig. 24 shows a cell of a twenty-two day old culture in the process of transformation from the fusiform to the round shape; it is moving around in the medium. Here a dark, yellowish endoplasm, filled with vacuoles and granulations, can be distinguished from a light, homogeneous ectoplasm. The latter is in constant wave-like motion. The changes in shape are continuous and very rapid in comparison with those of the fusiform cells. This particular cell wandered about in every direction, alternately approaching and leaving the fragment, but never continuing for long in the same direction.

Burrows found that the cells assumed a round form when they were placed into a medium containing evenly distributed products of metabolism. Nevertheless, the facts appear to us to argue that in addition to the chemical constitution of the medium account should also be taken of the consistency of the latter, in view of the fact that in liquid media round cells only were seen, and that the spindle-shaped cells in the non-liquid media always become spherical when surrounded by a small quantity of liquid. It may be suggested that the relation between the chemical constitution of the medium and that of the plasma on the one hand, and the relation between the consistency of the medium and that of the plasma on the other hand are also instrumental in determining the form of the cell. The point whether the round cells are normal or degenerate must depend on the length of time that they have ceased movement. Round cells which have been stationary for a long period of time doubtless degenerate as a result of the progressively deteriorating quality of the surrounding medium consequent upon metabolism. These are the round cells in which the nucleus is either pycnotic or entirely absent. At all events, it would be erroneous to designate the round cell *per se* as a degenerate form.

In this section we have seen that the shape of the cell is not an

active manifestation on the part of the cell, nor even connected with the cellular functions or with the origin of the cell (connective tissue or epithelium). The form of the cell is in no way connected with the morphologic character possessed by it while in the organism, which it introduces into the culture; on the contrary, the flatly polygonal epithelial cell can be made to assume a polyhedral, fusiform, or round shape, if the relation between the chemical constitution and the consistency of the medium on the one hand, and of the cell plasma on the other, be varied accordingly. Certain forms, such as the thread-like form of the columnar cells and of the posterior end of the bipolar spindle-shaped cells, are produced directly through tension.

We wish to mention that the method used by us possesses many disadvantages, which, however, we intend to rectify in our later experiments. It is above all necessary to utilize a medium which is either less exposed to a spontaneous variation of its consistency, or one at least in which this factor can be strictly controlled. In our subsequent investigations we shall endeavor to utilize media, the consistency and firmness of which may be accurately expressed in mathematical terms. Owing to the fact that our investigations bearing on this point are not yet sufficiently advanced, they can not here be taken into consideration.

Form and Function of the Cells.

If in our work with cultures we are able to observe how through external agents a change of form is imposed upon the epithelial cells, rendering them very similar to the connective tissue cells, it is but reasonable that these observations should be borne in mind when we return to an examination of the cells in the organism itself.

Hitherto the relations existing in the organism have not been investigated from this point of view; on the contrary, the study of the shape of cells and organs has been conducted from a standpoint according to which they are considered as an expression of cellular activity and as a consequence of cellular function. Moreover, the study of these relations in the organism itself, with exact methods subject to the control of the observer, is a problem much more difficult to compass.

Nevertheless, we believe that by observations allied to those above described we shall be led to a clearer understanding of the morphological character of the cell than is possible by the adoption of other methods which proceed from the theory of functional adaptation.

The phenomena observed in the cultures likewise obtain in the epithelia in the organism. For example, frog skin epithelium consists of a basal layer of cuboidal cells and of several layers of flatly polygonal cells. The situation of the former is similar to that of our polyhedral membrane cells; they are enclosed between two comparatively firm media, the connective tissue and the more or less tense flat epithelial cells. It is, therefore, not surprising that they possess a similar form. It may be that the remaining flat cells of the epithelium owe their form to the tension there prevailing, a condition which can not be similarly produced in the cultures by artificial means. The flat cells of the cultures always originate at places where the cells lie between two media of different firmness; in such cases they appear to be pressed flat to the firmer of the two media by means of adhesive forces. Flat cells of this type either adhere to the cover-glass (Harrison, Lambert, etc.), or, as observed in our own cultures, to the connective tissue. All the cells which remain attached to the connective tissue become flatly polygonal in shape. Nevertheless, in the organism cases are also seen to occur in which the epithelium is not under tension on all sides, but where the cells stray out freely while adhering to the connective tissue-like base. In such cases all the epithelial cells become flat in form, including those which were previously cuboidal in shape. This condition is always found in regenerating epithelial wounds (5). Here many epithelial cells also assume a spindle form, as they wander out from the edges of the wound into the wound scab, where they find similar conditions to those prevailing in the plasma medium. An abundant exudate in the wound, containing serum and fibrin, may furnish a medium more or less soft and conducting to this change. Leo Loeb (6) observed such spindle-shaped epithelial cells in the skin in process of regeneration in rabbits and guinea pigs.

The significance of these observations is apparent when they are compared with Champy's interpretation of his important observations made in connection with tissue cultures. Champy (2) states that the cells of some organs when

cultivated in blood plasma undergo certain changes which he calls "dedifferentiation." In cultures of the kidney, submaxillary, parotis, and thyroid from the fetal rabbit the cells of the glandular tubes soon begin to multiply actively through mitotic division. The minute canals sprouting from the cut tubules at first retain their epithelial character, but they immediately forfeit their character of gland cells. Finally, the basal membrane of these canals bursts and the epithelial cells mingle with the connective tissue cells of the interstitial tissue. At this stage they also lose their epithelial character and in conjunction with the connective tissue cells form a new tissue which is completely indefinite in character. Moreover, the interesting point in this connection is the fact that the indefinite tissue produced by all four organs is identical.

Such a process of "dedifferentiation," however, occurs only in organisms the cells of which undergo mitotic division. Therefore, it fails to occur in the organs of adult animals, which remain inactive outside of the organism. In the case of explants of cartilage, however, although mitotic division occurs, dedifferentiation fails to take place. The cells simply revert to a primitive condition, while at the same time they remain differentiated as cartilage cells.

Thus we find, as Champy himself realized, that for the "preservation of functional structure" function itself is by no means indispensable. For there are many organs the cells of which, failing to undergo mitotic division, preserve their so called "functional differentiation" when explanted. In this category we must include above all certain adult organs in which loss of functional structure would be expected to take place much earlier than in embryonal organs; that is, if the declarations of those who support the theory of functional adaptation be correct.² The cartilage, moreover, in spite of mitotic division, remains permanently cartilage, although of a primitive form, and in this case no atrophy from inactivity has occurred, as would have been a necessary presupposition in a case of functional adaptation. The case under consideration is a parallel of that of the transplanted salamander eyes. In these cases it was shown that although the animals were kept in the dark and had had their optic nerves cut, thus depriving the eyes of function and functional stimulus, these organs nevertheless failed to lose their functional structure (7). Thus far do the theories of Champy coincide with the established facts.

But he next concludes that the functional structure fails to arise in the newly formed cells owing to the culture's lack of functional stimulus. He bases his

² Roux' "functional period," which is supposed only to commence at an advanced, or at least, postembryonal stage.

theory on the following consideration. Before undergoing mitosis a cell first discards its differentiation. As the newly formed cells in the body are immediately subjected afresh to the influence of functional stimulus, they at once resume their functional structure; but in cultures which are devoid of all functional stimuli the reestablishment of the functional structure remains absent. Champy sets up this hypothesis immediately after observing that cartilage fails to dedifferentiate, although when cultivated its cells undergo mitotic division.

As a matter of fact the problem at issue in Champy's experiments must be looked for elsewhere. If every cell when about to undergo division renounces its functional structure and becomes embryonal, as has often been observed to be the case and as Champy himself admits, it is evident that the cells of the culture, even when exposed to functional stimuli, can never take on their original functional structure, as they continually proceed from division to division. It is therefore incorrect to assert that the absence of functional structure is to be ascribed to absence of function. The problematic point is rather the fact that the cells in the cultures never have a chance of developing. Were it possible for them to do so they would simply develop into their normal, inherited structures, among which are included the majority of characters which, according to Champy, are functional structures. That part of the structure, however, which owes nothing to the factor of inheritance is seen to arise passively in the cell, even in the absence of functional stimulus, as soon as any forces are brought to bear upon it (8).

We should like to recall the interesting experiments of Burrows in connection with heart muscle cells, as bearing on the question of the relation between development and differentiation in the cultivated cells. These heart muscle cells can be artificially reduced to a condition at which cell division takes place, at which stage they also undergo "dedifferentiation." If, however, the cell division is intercepted the cell is able to develop, and upon reaching the end of its development it begins to pulsate normally, independently of the operation of any specific functional stimuli.

It is, however, incomprehensible how, for instance, "lack of function" in the culture can be designated as the cause of the difference existing between the cultivated and the fetal parotid cells, in view of the fact that this lack of function exists in the body itself. For at the embryonic stage the parotid of the rabbit has not yet begun to functionate.

Finally, we must again revert to Champy's observations on the explanted cartilage.

Thoma (9) has repeatedly attempted to replace Roux' doctrine of functional adaptation by a new theory, which he designates as histomechanism and histochemistry. According to Thoma's histomechanical laws, cartilage can be formed from connective tissue only when the forces of pressure and tension have reached a certain maximum; as soon as they fall below this degree the cartilage is unable to subsist and reverts to connective tissue.

As has been recognized by Roux, this theory is fundamentally identical with the theory of functional adaptation; the difference is only one of name, as was shown in the case of the cartilage cultures. For it was seen that although the conditions of pressure prevailing in the plasma cultures differ essentially from those found in the body, the cartilage nevertheless continues to exist as such. Its cells by no means revert to the connective tissue type of cell, but they merely assume a more primitive character as a consequence of the mitotic divisions to which they are subjected. Here, therefore, the theory of functional stimulus is again at fault.

In conclusion we should like to call attention to another hypothesis of recent origin, by which it is sought to explain the variations in the morphological characters of the cultivated cells. We refer to Champy's so called theory of "inhibition" (10), a hypothesis no less obscure than that of functional adaptation.

Champy noticed that a tissue fails to dedifferentiate if it is explanted together with another "antagonistic" tissue, although when cultivated alone it rapidly undergoes dedifferentiation. When connective tissue and epithelium are cultivated together, the latter not only fails to dedifferentiate, but as in the cases of cicatrization of wounds, it grows around the connective tissue and remains a typical epithelium.

This observation of Champy's is accurate, and we have already reported similar findings in a previous communication (1). By the above described process we even succeeded in obtaining minute epithelial organisms, filled with connective tissue. But upon making an extensive examination of 600 cases we discovered that this result was but rarely obtained, and only in cases where the connective tissue was considerably firmer than the surrounding medium. In such cases the cells are pressed on to the connective tissue by means of simple

adhesion, just as under certain conditions the cells in a firm medium may be pressed on to the still firmer cover-glass. The fact that this results in their assuming a flat polygonal shape can certainly not be attributed to any inhibiting influences, but simply to the force by which they are being pressed against the base. Moreover, the statement that the epithelial cells always dedifferentiate as soon as they wander out into the medium is incorrect, for in firm media in which the epithelial cells migrate into the medium, they remain united in the form of an epithelial membrane and also preserve the typical form of the epithelial cell. The fact that the cells which in the body were flat in shape under these circumstances also assume a polyhedral form is to be ascribed to purely physical influences. Chanipy himself has shown that the cells remain united in the form of a membrane; but he believes that these cells are not epithelial, because the mitotic spindles are situated parallel instead of vertical to the plane of extension of the membrane. This latter fact we have observed to be correct (Fig. 5); nevertheless, it would be a mistake to assert that such a phenomenon never occurs in true epithelia, nor in the organism itself. Fig. 25 shows the regenerating epithelium in the tail of a tadpole (*Rana pipiens*). Such a regenerating epithelium shows many examples of mitosis in which the spindle is parallel to the surface of the cutis; we have one such example in our figure. No one would venture to state that regenerating epithelium is not true epithelium, and for that reason we must consider Champy's judgment to be inaccurate. The epithelium loses its inherent characteristics only when the cells are placed in a soft or semi-firm medium (according to our relative nomenclature), where the cytoplasm begins to disperse and the cells, now fusiform in shape, begin to separate, owing to the movements which are being initiated. Then only does it become hard to distinguish them from the ordinary connective tissue type of the cultures. Thus, the above processes are by no means caused by the suspension of some inhibiting influence, but simply through the physical changes in and around the cell.

The technique of tissue cultivation permits us to study the elementary parts of organs (the cells) with great exactitude. The more we are enabled, by means of our modern methods of technique, to pursue this detailed study, the more are we able to dispense with

the theories of "inhibiting influences" and "functional stimuli." By means of the above experiments in which we have shown that the form of the cell is dependent on the relation existing between the consistency of the medium and that of the cell plasma, we believe to have added some small contribution toward the attainment of this end. Changes in the consistency of these two substances, such as can be introduced into the culture by means of chemical procedures, result in a variation in the shape of the cell. Two additional factors of importance are, moreover, surface tension and adhesion, as was earlier shown by other investigators. But the effect produced by these latter must similarly depend upon the relative consistency of the medium and of the cell plasma. The next point of importance will be to establish the mathematical values of this relation, as applied to the various forms of cell. It is our intention to consider this aspect of the problem in subsequent investigations.

SUMMARY.

1. Fragments of skin from the leopard frog (*Rana pipiens*) were cultivated in media of varying consistency. A mixture of frog plasma, frog muscle extract, chicken plasma, and chicken embryo extract usually produced a very firm medium; a mixture of frog plasma, frog muscle extract, and chicken plasma, one less firm (semi-firm); and a mixture of frog plasma and frog muscle extract a medium of a consistency varying from soft to liquid.

2. (a) In a firm medium the cells which migrate into the medium are polyhedral (polygonal when seen from above) in form, which shape they retain permanently. They remain united in a compact membrane, the central parts of which consist of several (three to four) layers of cells. Migration of isolated cells into the medium does not take place.

(b) In the semi-firm media the cells situated at the edge of the membrane become fusiform in shape, gradually detach themselves from the membrane, and stray out individually into the medium. This causes the membrane to become loose in character, and to contain holes, while its edges at the same time become very irregular and send out pointed projections.

(c) In a soft medium the cells are fusiform or thread-like in shape. The migration of isolated cells is much more pronounced than in the semi-firm media, as a result of which the membrane undergoes constant and rapid loosening up. By this means whole portions of the membrane become detached and their separate parts are at first united by the thread-like columnar cells, which become drawn out in the form of long threads upon the separation of the individual sections of the membrane. The loosening up of the membrane is further assisted by liquefaction and the consequent formation of vacuoles; the latter process likewise results in the formation of thread-like columnar cells.

(d) Liquid media contain only round cells.

3. This serves to explain numerous internal processes of the organism, especially certain changes of form observed by Leo Loeb in transplantations of wound scabs and of skin; the conditions artificially produced by Leo Loeb must have effected a change in the consistency of the medium.

4. It has been shown that it is unnecessary, for a satisfactory explanation of the above findings, to have recourse to the theories of "functional stimulus" or "inhibiting influences," by means of which Champy wished to account for the variations in the morphological character of the cells.

I wish here to acknowledge my indebtedness to Mr. Louis Schmidt, for the care with which he has made the photographic reproductions of my cultures. Mr. Schmidt has succeeded in working out a new method for counteracting the destructive effects of the condensation of water which is apt to occur in the cultures when exposed to the rays of the arc light after having been previously kept at a low temperature (21° to 23° C.).

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EXPLANATION OF PLATES.

PLATE 6.

FIG. 1. Frog skin, cultivated in a firm medium (F.Pl. + F.E. + Ch.Pl. + Ch.E.); about twenty hours after explantation. A dense membrane of polygonal epithelial cells surrounds the old fragment. A small vacuole still exists on the right side only, which has caused some cells to become elongated. Culture B 174; Giemsa stain; magnification $\times 30$.

FIG. 2. Frog skin, cultivated in a semi-firm medium (F.Pl. + F.E. + Ch.Pl.); three days after explantation. Part of the cell membrane, fairly remote from the border of the membrane, where the latter still consists of two to three layers of cells. As shown by this cross-section, they are cuboidal in shape; as shown in Fig. 8, seen from above, they are polygonal. Therefore their actual shape is polyhedral. Camera lucida drawing; Culture 7. Cross-sections stained with Giemsa solution after fixation in potassium bichromate, 70 per cent; glacial acetic acid, 20 per cent; formalin, 10 per cent; magnification, Leitz oc. 3; obj. 1/12, oil immersion; tube 170.

FIG. 3. Frog skin, cultivated in a firm medium (F.Pl. + F.E. + Ch.Pl. +

Ch.E.); about twenty hours after explantation. A piece of the border of the cell membrane, seen from above, where the cells have a polygonal shape. The spaces between the cells are artificially produced. Camera lucida drawing. Culture B 169. *In toto* preparation, stained with Giemsa solution after fixation in formalin 2 per cent; magnification, Leitz oc. 3; obj. 6; tube 160.

PLATE 7.

FIG. 4. Frog skin, cultivated in a firm medium (F.Pl. + F.E. + Ch.Pl. + Ch.E.); six days after explantation. A dense membrane of polygonal cells (epithelial) surrounds the original fragment. No cells migrate out of the membrane into the medium. Culture B 114; Giemsa stain; magnification $\times 30$.

FIG. 5. Border of the cell membrane of the same culture as seen in Fig. 4, highly magnified. The shape of the cells is shown in a firm medium; they are polygonal, even those situated at the edge of the membrane. Two of the cells are undergoing mitotic division. Culture B 114; Giemsa stain; magnification $\times 1,000$.

PLATE 8.

FIG. 6. Frog skin, cultivated in a firm medium (F.Pl. + F.E. + Ch.Pl. + Ch.E.); ten days after explantation. A dense membrane of polygonal epithelium-like cells surrounds the original fragment. The border of the membrane is uniform, and even the border cells are polygonal in shape. No cells are migrating from the membrane into the medium. Culture B 103; photographed while alive; magnification $\times 30$.

FIG. 7. Frog skin, cultivated in a semi-firm medium (F.Pl. + F.E. + Ch.Pl.); about twenty hours after explantation. The cell membrane is dense only in the center; at the edge of the fragment it is loose, and here large pointed tongues of tissue project out into the medium. At the tips many spindle-shaped cells are in the act of becoming detached from the membrane and migrating into the plasma. Most of the cells situated at the edge have changed from being polygonal to fusiform. Therefore, the edges of the membrane are serrated, the medium being filled with free spindle-shaped cells. Culture B 86; Giemsa stain; magnification $\times 30$.

PLATE 9.

FIG. 8. Frog skin, cultivated in a semi-firm medium (F.Pl. + F.E. + Ch.Pl.); three days after explantation. The same characteristics are seen as in Culture B 86 (Fig. 7), but more pronounced. There are many holes in the membrane at the back of the projections, produced by the forward motion of the cells into the medium, and they are not filled with fluid. Culture B 7; photographed while alive; magnification $\times 60$.

FIG. 9. The polygonal cells at the edge of the cell membrane have assumed a spindle shape and become isolated from the membrane when cultivated in a soft medium. Drawing, made with a magnification, Leitz oc. 3; obj. 6; but without camera lucida.

FIG. 9 a. Culture A 406 c, cultivated in F.Pl. + F.E., six days after explantation. I. The homogeneous ectoplasm flows out into the medium. II. Cells a

and b assume a flask-like shape. III. Cell a has become isolated and contracted. IV. Cell a resumes the fusiform shape.

FIG. 9 b. Culture B 9, cultivated in F.Pl. + F.E. + Ch.Pl., four days after explantation. I. Cell a moves out into the medium, but remains connected by a thin plasma bridge with the other cells. II. Cell a assumes a round shape. III. Cell a resumes the spindle shape.

PLATE 10.

FIG. 10. Frog skin, cultivated in a semi-firm medium (F.Pl. + F.E. + Ch.Pl.) ; twenty-one days after explantation. Only the most central zone of the membrane is compact, the borders are drawn out into long cloven tissue tongues, giving the culture a star shape. The migration of isolated spindle cells is considerable, and more extensive than in Figs. 7 and 8. Culture B 78; photographed while alive; magnification $\times 30$.

PLATE 11.

FIG. 11. The same culture as in Fig. 10, but twenty-two days after explantation. The cell membrane has further developed in size; there are long tissue projections and large holes in the cell membrane, some of which have been filled up with a softer medium. The hole in the upper part of the figure especially is bridged over by thread-like columnar cells. There are large numbers of isolated, migrating cells around the cell membrane, which spread out far into the medium. Culture B 78; Giemsa stain; magnification $\times 30$.

PLATE 12.

FIG. 12. Spindle-shaped cells of epithelial origin in a culture of frog skin, cultivated in a semi-firm medium (F.Pl. + F.E. + Ch.Pl.) for twenty-two days (same culture as in Fig. 11); the cells are migrating from the border of the tissue projections into the medium.

FIG. 12 a. Tip of a tongue of cells, projecting from the membrane into the medium, where the cytoplasm of the originally polygonal cells is seen flowing out into the medium, by means of which process the cells are assuming a spindle-like shape. The nuclei are also stretched out and elongated, and have a clear nuclear plasma and in addition one or two nucleoli, but no chromatin net, as is always the case in the polygonal cells, when fixed in formalin; the cytoplasm is more or less foamy in structure.

PLATE 13.

FIG. 12 b. Two spindle cells shortly after isolation from the membrane, still connected with each other. Both possess some vacuoles in the cytoplasm, one of which is distinctly differentiated into a clear ectoplasm and a foamy endoplasm.

FIG. 12 c. Isolated and moving spindle cell. The anterior end, directed to the external edge of the drop of medium, is stretched out into a long, thin process, at the end of which a slight swelling has developed.

PLATE 14.

FIG. 13. Frog skin, cultivated in a soft medium (F.Pl. + F.E.); about twenty hours after explantation. Nearly the whole membrane is migrating away from the old fragment into the medium, in which we see little pieces of tissue moving freely. Holes of various sizes have developed in consequence of the migration of the border cells, the external parts of the membrane being connected with the internal ones by means of the thread-like columnar cells, which are long drawn out. The holes are not filled with a medium of different consistency from that prevailing outside, both being soft but not liquid. Nearly all the cells of the membrane, including the internal ones, are elongated; the cells at the edge are spindle-like. Culture B 119; photographed while alive; magnification $\times 30$.

PLATE 15.

FIG. 14. Frog skin, cultivated in a soft medium (F.Pl. + F.E.); six days after explantation. Similar to Fig. 13. Isolated spindle-shaped cells and thread-like columnar cells can plainly be seen. Culture B 121; stained with Giemsa solution after fixation in formalin 2 per cent; magnification $\times 30$.

PLATE 16.

FIG. 15. Frog skin, cultivated in a medium which is partly firm, and partly in the act of liquefying (F.Pl. + F.E.); four days after explantation. Large vacuoles are causing the cells to become stretched out, thus producing thread-like columnar cells. In the firm part of the medium a compact cell membrane is seen. The upper layers are separated from the fragment and form a thin membrane, which covers a part of the old fragment and part of the compact cell membrane. No change has been going on in these upper layers, the cells of which are neither growing nor moving. Culture B 117; photographed while alive; magnification $\times 30$.

FIG. 16. Frog skin, cultivated in a soft medium (F.Pl. + F.E.); eighteen days after explantation. Only a very small, thin membrane remains around the old fragment. This membrane is extended into several tissue projections, from which the spindle-shaped cells are becoming separated and are migrating into the medium. The medium contains numbers of isolated cells, which are spindle-like or round. Culture B 77; photographed while alive; magnification $\times 30$.

PLATE 17.

FIG. 17. Frog skin, cultivated in a soft medium (F.Pl. + F.E.), which had almost completely liquefied when the photograph was taken twenty-one days after explantation. The old fragment has become a globular ball. The cell membrane has entirely loosened up; in its place there are only isolated cells, most of which are round, but some are spindle-shaped. Culture B 81; photographed while alive; magnification $\times 30$.

PLATE 18.

FIG. 18. A small fragment of a cell membrane, grown out of a piece of frog skin in a firm medium, isolated five days after explantation and kept for

two days in a firm medium (F.Pl. + F.E. + Ch.Pl. + Ch.E.), at which time the picture was taken. A dense, compact membrane has grown out of the original fragment of membrane, consisting of polygonal cells. No migration of isolated cells into the medium is taking place. Culture B 100 a; photographed while alive; magnification $\times 30$.

FIG. 19. A fragment of a cell membrane, grown out of a piece of frog skin in a soft medium, isolated four days after explantation, and kept in a soft medium (F.Pl. + F.E.) for about twenty hours, at which time the picture was taken. A network of elongated, spindle-shaped, and thread-like cells has developed from it, which greatly resembles connective tissue. The thread-like columnar cells are clearly seen in this photograph. Culture B 119 a; stained with Giemsa solution after fixation in 2 per cent formalin; magnification $\times 30$.

PLATE 19.

FIG. 20. Frog skin, cultivated in an unusually firm F.Pl. + F.E. medium. It possesses the same characteristics as a culture in a firm F.Pl. + F.E. + Ch.Pl. + Ch.E. medium (compare Figs. 1, 4, and 6). Culture A 157; about twenty hours after explantation; Giemsa stain, after fixation in 2 per cent formalin; magnification $\times 30$.

FIG. 21. Frog skin, cultivated in an unusually firm F.Pl. + F.E. + Ch.Pl. medium; two days after explantation; the cell membrane and its cells, grown out of the old fragment, have the same characters as in a firm (F.Pl. + F.E. + Ch.Pl. + Ch.E.) medium (compare with Figs. 1, 4, and 6). Culture B 13; photographed while alive; magnification $\times 30$.

PLATE 20.

FIG. 22. Frog skin, which was cultivated in a firm medium (F.Pl. + F.E. + Ch.Pl. + Ch.E.); the membrane had for ten days the compact character peculiar to a firm medium. On the eleventh day the fresh medium, although of the same composition, had become softer and the membrane and the cells at once assumed the characteristics of cultures kept in semi-firm media of the composition of F.Pl. + F.E. + Ch.Pl., as is shown in this photograph (compare Figs. 7, 8, and 11). Culture B 100; eleven days after explantation; stained with Giemsa solution after fixation in 2 per cent formalin; magnification $\times 30$.

PLATE 21.

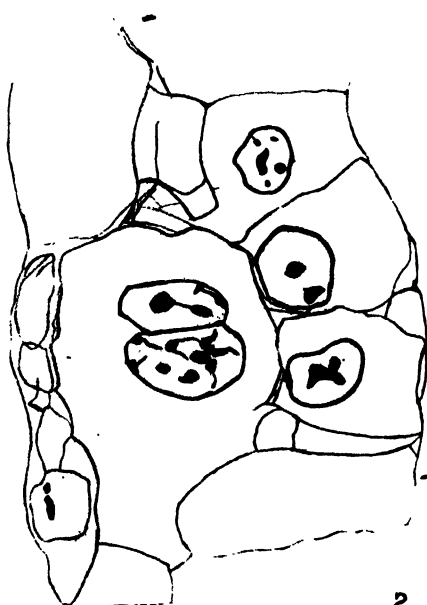
FIG. 23. Round cells. Fig. 23 a and b, from Culture B 81, which was placed in a liquid medium (compare Fig. 17), consisting of F.Pl. and F.E.; Fig. 23 c, from a semi-firm medium, consisting of F.Pl. + F.E. + Ch.Pl. + Ch.E.; fixed in formalin 2 per cent; Giemsa stain; camera lucida drawing; magnification, Leitz oc. 3; obj. 1/12, oil immersion; tube 170.

FIG. 24. Various stages of the movement of a formerly spindle-shaped cell, which is in process of becoming round; a twenty-two day old culture of frog skin, kept in a medium which is becoming liquid (F.Pl. + F.E. + Ch.Pl.). Culture B 45; drawing made from the living specimen; magnification, Leitz oc. 3; obj. 6; tube 160; schematic.

FIG. 25. Skin epithelium of a regenerating tail of the tadpole of *Rana pipiens*, showing a mitosis, the spindle of which is parallel with the surface of the connective tissue. Taken one day after the tip of the tail had been cut; fixed in potassium bichromate, 70 per cent; glacial acetic acid, 20 per cent; formalin, 10 per cent; stained with iron hematoxylin. Camera lucida drawing; half size; magnification, Leitz oc. 3; obj. 6; tube 160.



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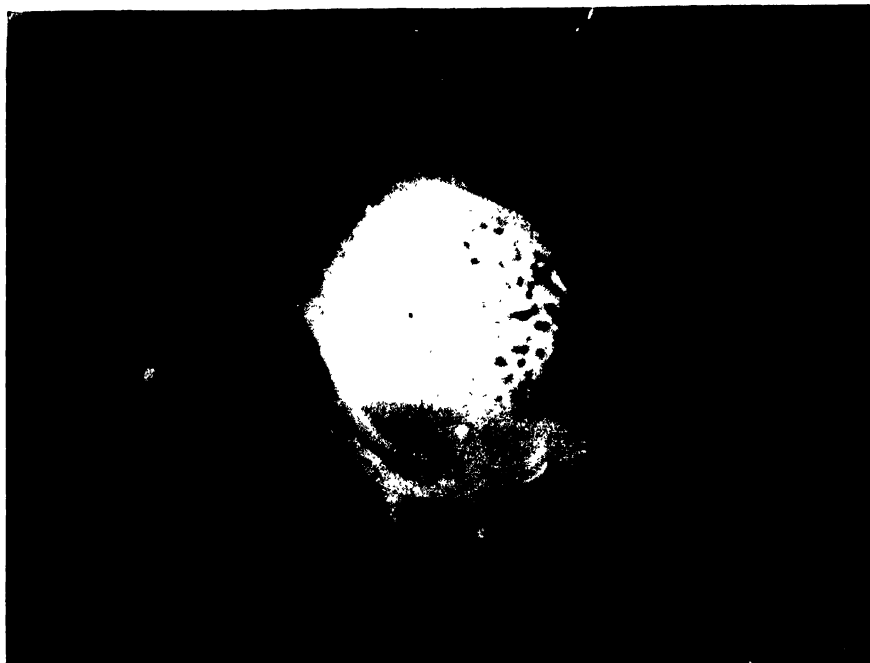


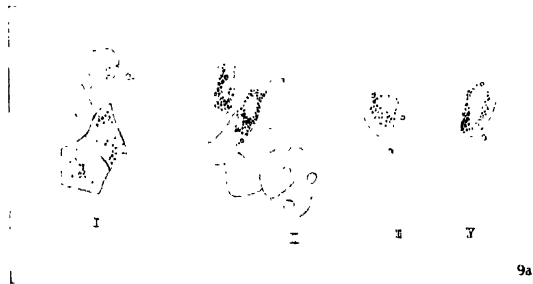
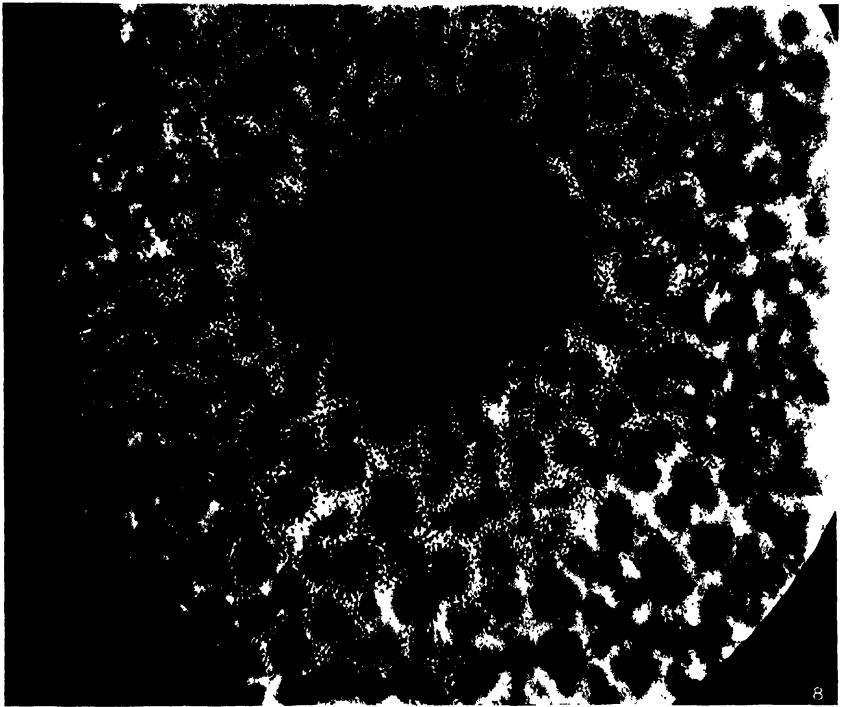
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(Uhlenhuth: Epithelial Cells in Cultures of Frog Skin.)

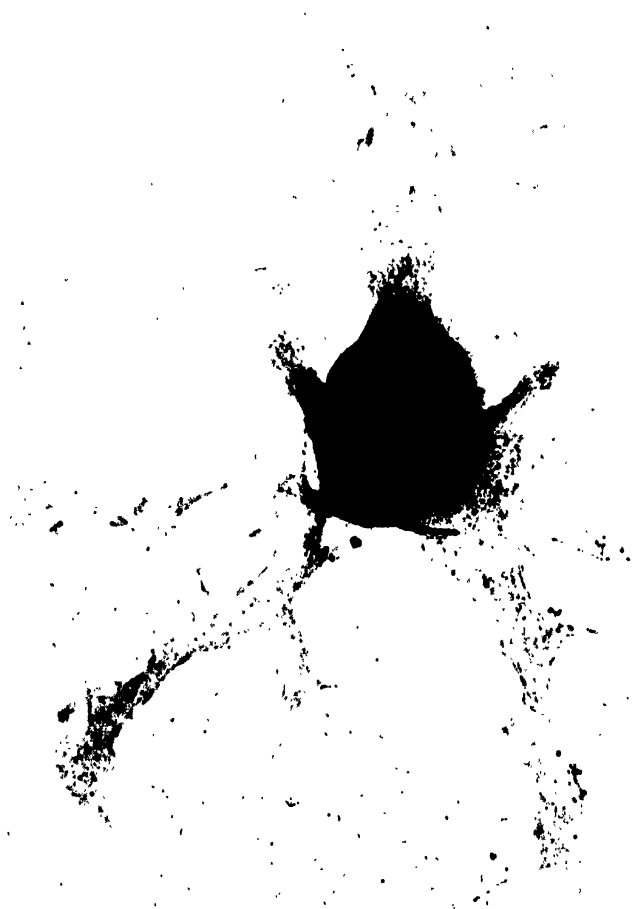




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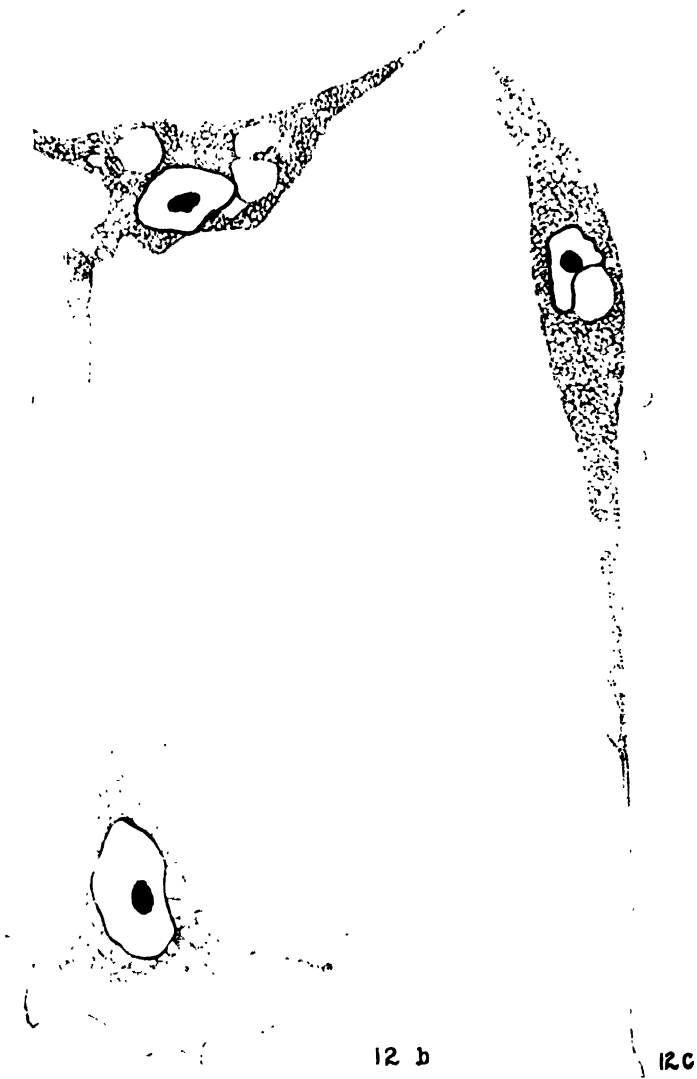


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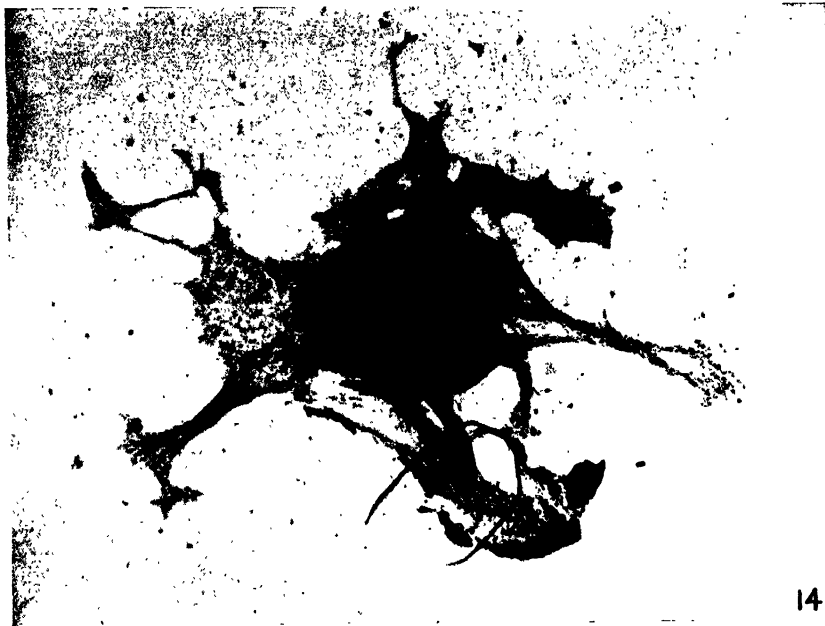


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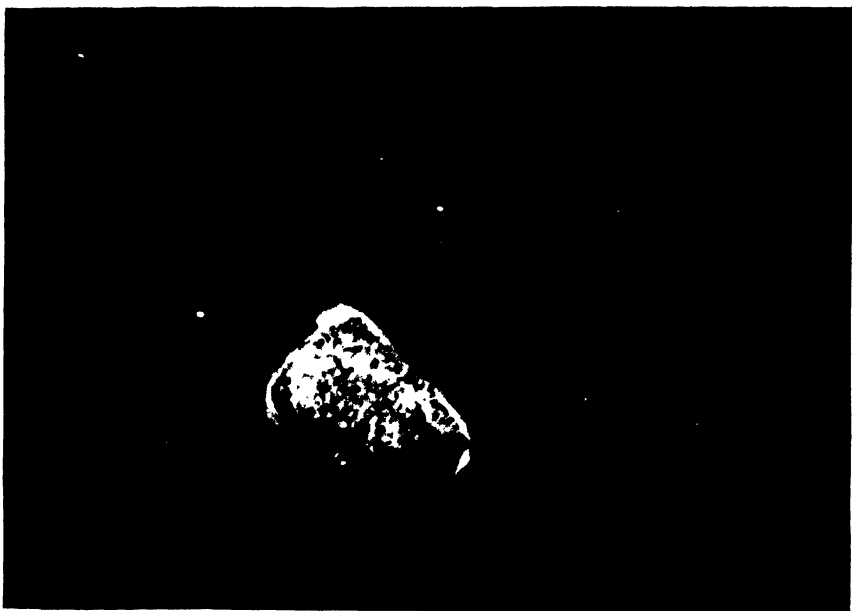


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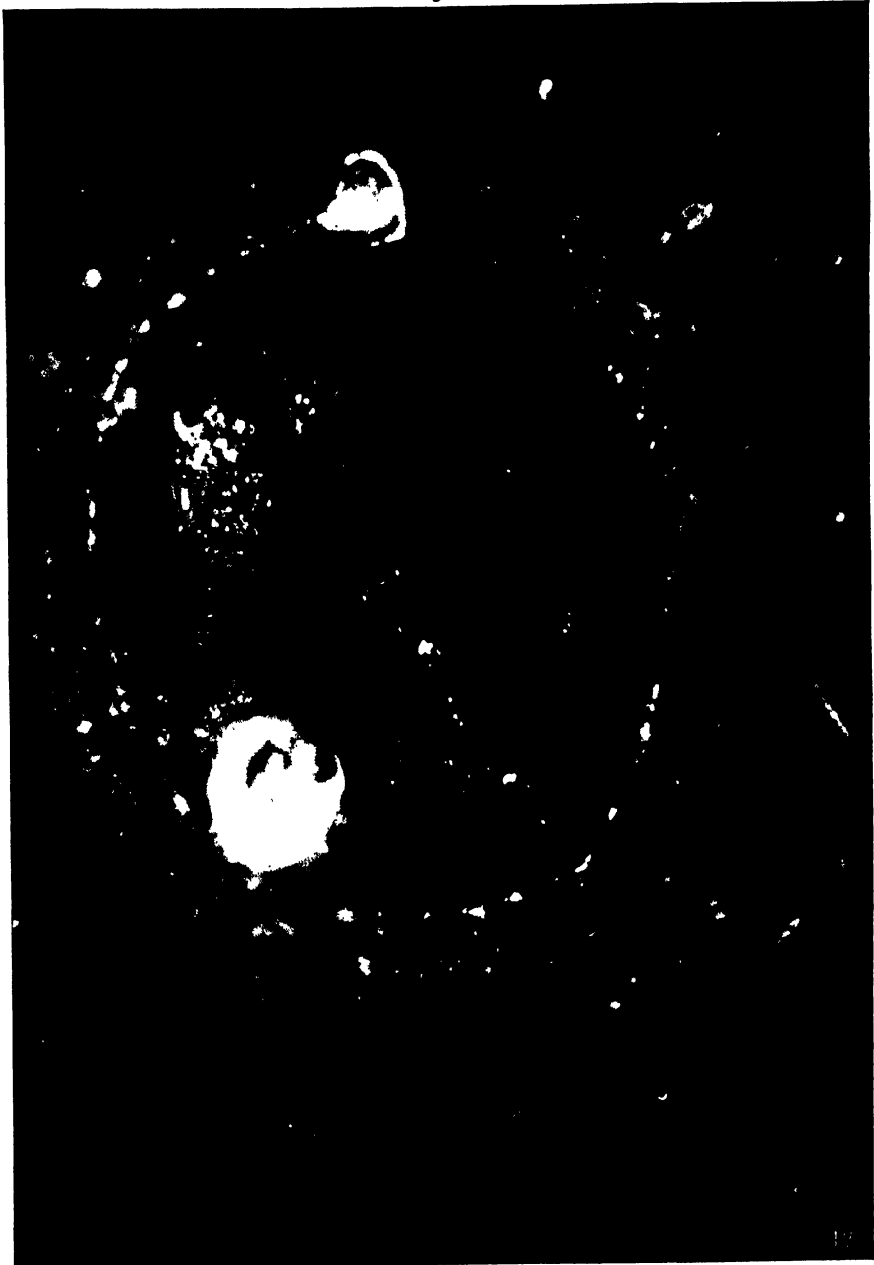
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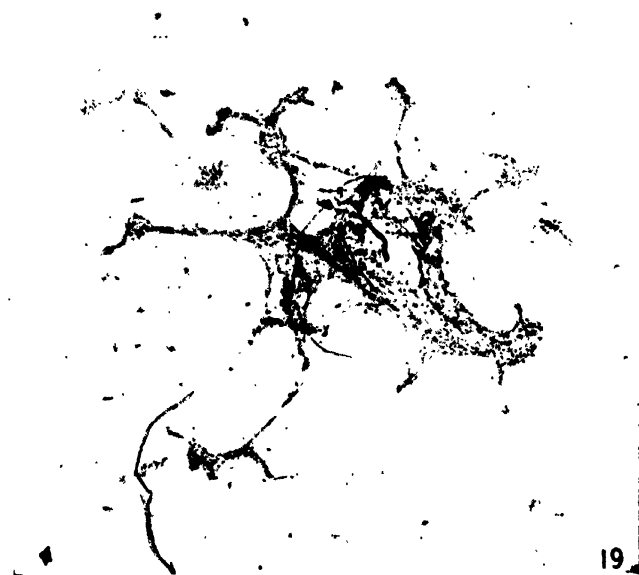
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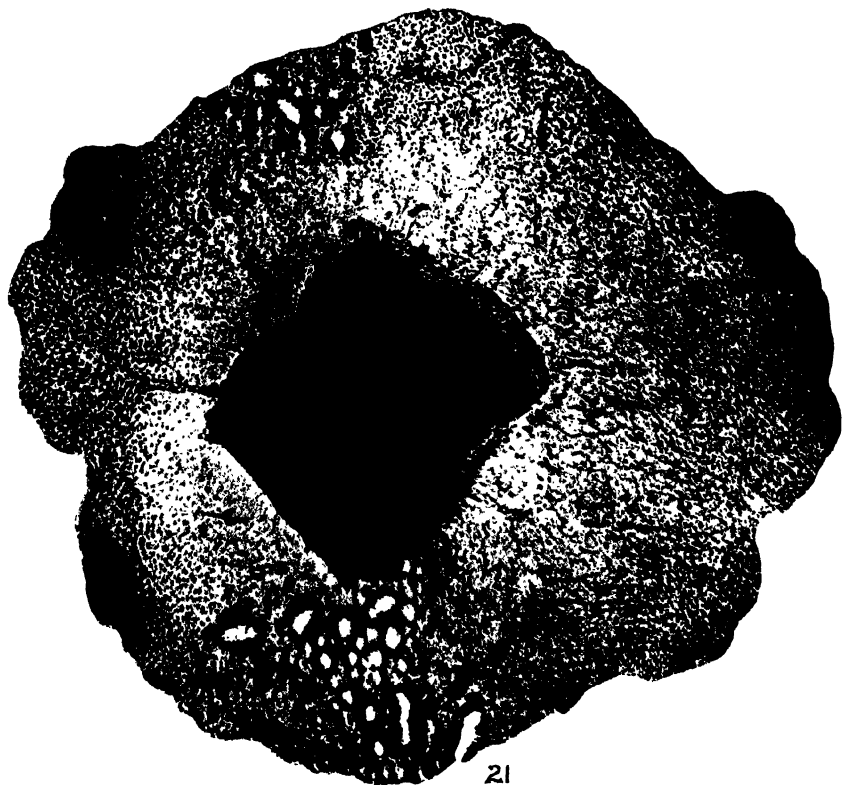
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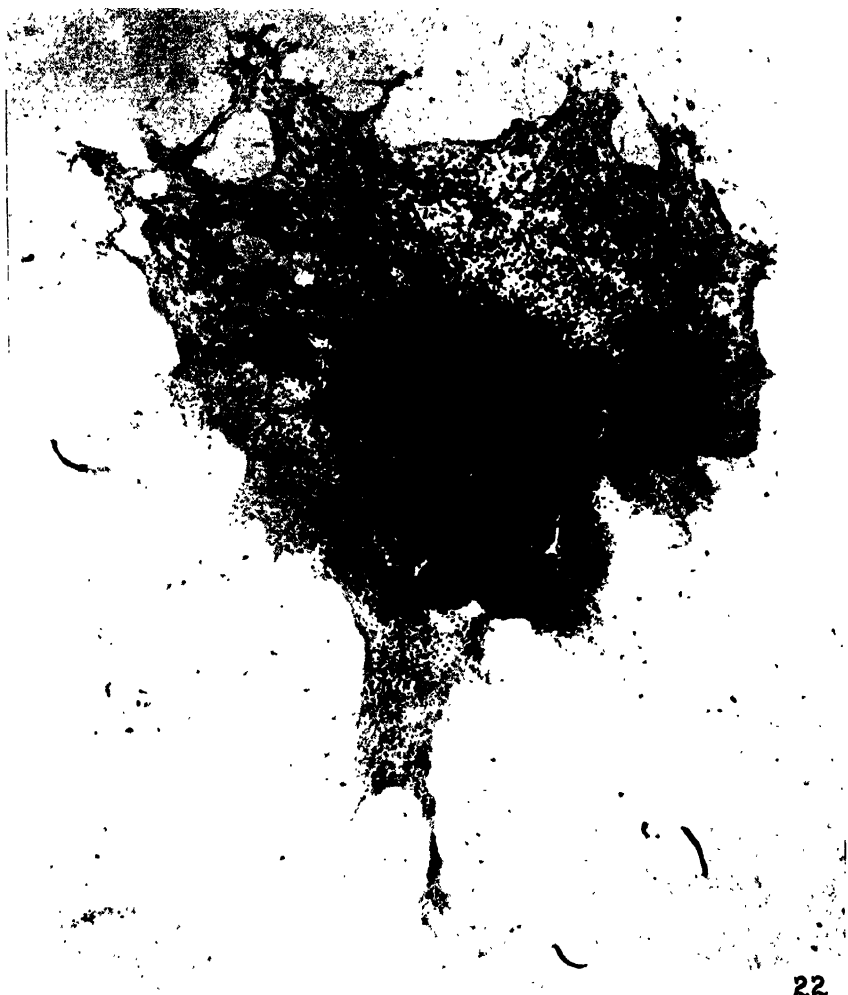
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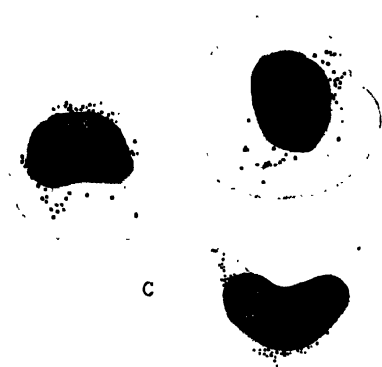


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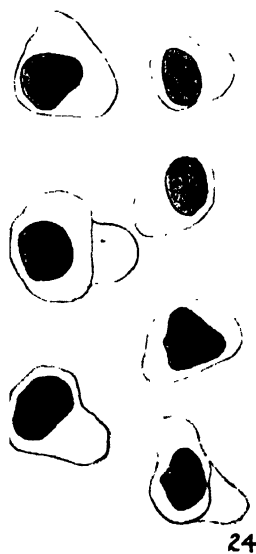
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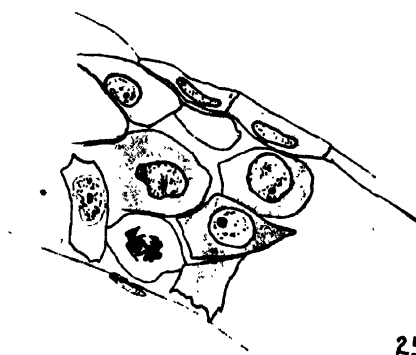
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25

ON THE NATURE OF THE CONDITIONS WHICH DETERMINE OR PREVENT THE ENTRANCE OF THE SPERMATOZOON INTO THE EGG.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

I.

The well-known fact that a spermatozoon can no longer enter an egg after it is once fertilized raises the question whether this is due to the changes necessarily connected with development; or whether development of an egg can take place without the existence of such a block. We are in possession of facts speaking in favor of the second view. Thus the writer has shown that if the eggs of *Strongylocentrotus purpuratus* or *Arbacia* are induced to develop by the methods of artificial parthenogenesis a spermatozoon can enter the egg or an individual blastomere of a segmenting egg, while the latter is in the full process of development. This leaves no doubt that the block caused by the entrance of a spermatozoon into an egg for the entrance of further spermatozoa must be due to a change not necessarily identical with that inducing the development of the egg.

A second group of observations made by the author deals with the phenomena of specificity and these prove that the block which an egg offers to heterogeneous sperm is rapidly reversible and confined to the surface of the egg or the spermatozoon or both. In the case of the egg of *purpuratus* and sperm of *Asterias* (and many similar instances) the specific block can be overcome if we slightly increase the alkalinity of the sea water. The spermatozoon can only enter the foreign egg while both sperm and egg are in the hyperalkaline sea water, whereas if the egg and sperm are treated separately with hyperalkaline solution (no matter how long) and put together in a sufficiently large quantity of normal sea water no egg can be fer-

tilized¹ while fertilization will take place as soon as the hyperalkalinity is restored. This shows that the change (brought about by the hyperalkaline sea water) which makes the fertilization possible is rapidly reversible, as we should expect it to be if it consisted merely in a physical change at the surface of the egg. To this series of facts, others might be added which point in the same direction. In this paper we intend to discuss a little more fully the various conditions which block or favor the entrance of a spermatozoon into an egg, in order to form an idea of the nature of the forces which control these phenomena.

II.

I. When the unfertilized eggs of *S. purpuratus* are treated for two hours with hypertonic sea water (50 c.c. sea water + 8 c.c. $2\frac{1}{2}m$ NaCl or Ringer solution) the eggs of certain females² will develop into blastulæ, gastrulæ and plutei, while the eggs of other females can not be caused to develop in this way. These individual differences coincide possibly with those observed by the writer in regard to spontaneous membrane formation in the eggs of different females³ and it is possible that only the eggs of such females of *purpuratus* can be induced to form larvæ through a mere treatment with hypertonic sea water in which the latter can induce the cortical changes underlying the membrane formation. Whatever the nature of the individual difference may be, *purpuratus* eggs which have been induced to develop into larvæ by a hypertonic solution can be fertilized with sperm while they are in the process of segmentation. When such eggs are in the two-, four-, eight-, or sixteen-cell (and possibly also later) stages the sperm can enter into one or more blastomeres of such an egg and this entrance betrays itself by a distinct and clear membrane formation around each blastomere.³ While the segmenting eggs which were not fertilized with sperm develop into larvæ,

¹ The large quantity of sea water is necessary so that the hyperalkaline sea water at the surface of the egg and sperm can diffuse away before both gametes come in contact.

² Loeb, *Arch. f. Entwicklungsmech.*, XXXVI, 626, 1913; "Artificial Parthenogenesis and Fertilization," Chicago, 1913, p. 219.

³ Loeb, "Artificial Parthenogenesis and Fertilization," p. 240; *Arch. f. Entwicklungsmech.*, XXIII, 479, 1907.

those into which sperm enters perish very rapidly. This simple and rather striking experiment which can easily be performed in the eggs of *Strongylocentrotus*, where the membrane formation around a single blastomere can be clearly recognized, shows that the process of development in a fertilized egg in itself can not be responsible for the block caused by fertilization. It looks as if the entrance of a spermatozoon into the mature egg, independently of the developmental changes it induces in the egg, causes some physical or physico-chemical change (of the surface of the egg?) which renders the subsequent entrance of a spermatozoon impossible.

2. With the eggs of most females of *purpuratus* the treatment with a hypertonic solution does not lead to a development into larvæ, but only to the first segmentation stages in a limited number of eggs (provided that the eggs have been exposed to the solution the proper period of time). Such blastomeres afterwards go into a resting stage. If one waits long enough, until there is no doubt left that the blastomeres have reached a resting condition and will divide no further, and if one then adds sperm, the individual blastomeres can again be fertilized, which is indicated by a membrane formation around each individual blastomere and the subsequent development of such blastomeres into swimming larvæ.⁴ The fact that each individual blastomere in this case is fertilized independently of its neighbors suggests that there is no protoplasmic connection between the neighboring blastomeres; otherwise the entrance of a spermatozoon into one should cause its neighbors also to form a fertilization membrane, which does not happen.

All these facts show that the changes underlying development do not necessarily prevent the entrance of a spermatozoon into an egg fertilized by sperm.

3. Development can be initiated in an unfertilized egg by causing a membrane formation by a fatty acid. Eggs after such an artificial membrane formation perish as a rule rapidly at room temperature (if no second treatment is given them) but they may segment if kept at a low temperature. The eggs are usually put after treatment with the butyric acid into normal sea water in which they form a membrane. This membrane is different in the eggs of

⁴ Loeb, *Arch. f. Entwicklungsmech.*, XXIII, 479, 1907; "Artificial Parthenogenesis and Fertilization," p. 237.

different species of sea urchins. In the egg of *S. purpuratus* the membrane is tough and entirely impermeable to the spermatozoon. When we add sperm to such eggs with a butyric acid membrane they behave exactly as if no sperm had been added, they all perish rapidly (at room temperature). The question arose, if a spermatozoon could still enter the egg of *purpuratus* after membrane formation, provided the membrane could be destroyed. This can be done in a certain percentage of the eggs of *purpuratus* by shaking them after artificial membrane formation; the number of eggs whose membrane is torn varies in different experiments owing probably to differences in the thickness and toughness of the membrane. Even if the membrane is torn the edges may come close together again so that the opening often is closed again and no spermatozoon can go through. Kupelwieser and the writer performed this experiment on the eggs of *purpuratus* and it was found that such eggs with torn membranes were fertilized upon the addition of sperm and developed normally; while the eggs whose membranes were intact all perished.⁵

The writer repeated this experiment last winter with the same result. He found that eggs with torn membranes when subsequently fertilized with sperm did not form any new membranes as he had stated before. It is possible that he mistook at that time the new hyaline membrane which forms around the egg after membrane formation and fertilization for a new fertilization membrane.

It is not necessary that these eggs be fertilized immediately after the artificial membrane formation, the experiment succeeds also after some time (one hour or more); only with this difference that the eggs perish very rapidly after the membrane formation if they receive no second treatment. In order to avoid this difficulty the writer last winter proceeded as follows: Artificial membrane formation was produced in the eggs of a *purpuratus* and all eggs had formed perfect membranes. One control was kept and the rest were shaken. These were divided into three lots, one served as a control; the eggs of the latter all perished as fast as the eggs of the first control (which were not shaken). The second lot were fertilized after about one half hour after membrane formation.

⁵ Loeb, "Artificial Parthenogenesis and Fertilization," p. 234.

Twenty per cent. of these eggs developed into normal larvæ, the rest perished. The percentage of developing eggs corresponded roughly with the percentage of eggs whose membrane was torn. The third lot of the shaken eggs was put overnight into 50 c.c. sea water + 7 drops of 1/10 per cent. KCN, to prevent the disintegration of these eggs. The next morning (sixteen hours after the membrane formation) the eggs of Lot 3 were transferred into normal sea water and divided into two lots, one was fertilized with sperm, the other was kept as a control. About twenty per cent. of the eggs which were fertilized began to segment, but many in an abnormal way and none developed into larvæ. Of the second lot to which no sperm was added also a few began to segment. As the writer has shown in former experiments, the eggs of *Strongylocentrotus* can be caused to develop after artificial membrane formation if they are either treated for a short time with a hypertonic solution or if for a longer period the oxidations are suppressed in them by lack of oxygen or the addition of cyanide. There is therefore no doubt that the eggs of *purpuratus* in which the artificial membrane formation has been induced by butyric acid can be fertilized subsequently with sperm.

4. The treatment of the eggs of *Arbacia* with butyric acid leads to the formation of a membrane which varies considerably in the eggs of the same female. Some eggs have a thin membrane which is permeable to the spermatozoon, others have a tough fertilization membrane which is as impervious to the spermatozoon as the regular fertilization membrane. The percentage of the eggs with membranes permeable for sperm varies very much in different experiments, according to the material and according to the external conditions. If this is kept in mind it is easily understood that the number of *Arbacia* eggs which can be fertilized after they have been treated with butyric acid differs in different experiments. Since the membrane called forth by butyric acid is not always plainly visible, it is a prerequisite that always one set of such eggs should be set aside as controls to ascertain whether or not *all* the eggs disintegrate rapidly (if no second treatment is given to them). Only if they all disintegrate rapidly have we any guarantee that in all of them the membrane formation has been effective. The former experiments

of the writer show that such eggs can be fertilized by sperm; in fact they show that while the unfertilized eggs disintegrate rapidly after the inducement of the membrane formation with butyric acid, the subsequent fertilization of such eggs by sperm saves their lives and makes them develop.⁶

III.

1. It is a well-known fact that most eggs can only be fertilized by sperm of their own or a closely related species. The writer thought that in order to obtain light on the nature of the block to the entrance of heterogeneous sperm it was necessary first to find the means by which this block could be overcome. He succeeded in showing that the egg of the sea urchin *S. purpuratus* can be fertilized by the sperm of starfish, brittle stars, and holothurians in sea water (or other balanced solutions) if their alkalinity was a trifle higher than that of ordinary sea water (*e. g.*, in a solution of 50 c.c. sea water + 0.6 c.c. *N/10* NaOH).⁷ Godlewski⁸ succeeded by the same method in the fertilization of the egg of the sea urchin with the sperm of crinoids.

The most important fact found out in this connection was the following, namely, that the fertilization of the egg of *purpuratus* by the sperm of *Asterias* only takes place while both eggs and sperm are in this hyperalkaline solution. If eggs and sperm are put into these solutions separately and if then from time to time sperm and eggs so treated are transferred into normal sea water, as a rule not a single egg is fertilized; while with the same material when eggs and sperm are together in the hyperalkaline solution as many as 100 per cent. of the eggs may be fertilized. The effect of the alkali is, therefore, rapidly reversible; the eggs when put from the hyperalkaline sea water free from sperm into the normal sea water containing very motile sperm of *Asterias* can not be fertilized; when put back into hyperalkaline sea water containing *Asterias* sperm they will be fertilized rapidly.

⁶ Löeb, *Arch. f. Entwicklungsmech.*, XXXVIII, 416, 1914.

⁷ Loeb, *Pflüger's Arch.*, XCIX, 323, 1903; CIV, 325, 1904; *Arch. f. Entwicklungsmech.*, XL, 310, 1914; *Science*, N. S., XL, 316, 1914.

⁸ Godlewski, *Arch. f. Entwicklungsmech.*, XX, 579, 1906.

This rapid reversibility of the effect of the NaOH indicates that it must be confined to the surface of the egg and the spermatozoon or both; and this is corroborated by the fact that the NaOH does not enter the cells. One of the forces which determine the entrance of the spermatozoon into the egg may be surface tension and the phenomenon of the entrance may be comparable or possibly identical with the phenomenon of phagocytosis.

Godlewski mentioned that he occasionally observed a fertilization of the egg of the sea urchin with the sperm of a crinoid in normal sea water after both had been treated with hyperalkaline sea water separately. This observation is correct but finds its explanation in the assumption that in such cases the hyperalkaline sea water had not had time to diffuse from the jelly of the egg or from the surface of the egg protoplasm by the time the spermatozoon came in contact with it. In order to test this view the writer treated the eggs of *purpuratus* with a hyperalkaline solution of greater than the optimal concentration while the sperm was treated separately with the optimal concentration (50 c.c. sea water + 0.6 c.c. $N/10$ NaOH) and then both were mixed in a little sea water in a watch glass. In such a case a large number of eggs were fertilized, but while the fertilization occurred nominally in normal sea water it really occurred in a layer of hyperalkaline sea water surrounding the protoplasm of the egg.

The conclusion from these experiments is that the block to the entrance of the spermatozoon of *Asterias* into the egg of *purpuratus* is of a rapidly reversible character, consisting in some alteration of a physical property of the surface. On this assumption the factor of specificity consists of an agency which affects these properties of the surface of the egg in the same sense as the increase in the concentration of the alkali. It should be added that the writer observed also that an increase of the concentration of Ca in the sea water acts in the same sense as an increase in the alkalinity; and that if the concentration of Ca is increased the increase of NaOH may be less than is necessary otherwise.

2. If the idea was correct that the factor of specificity contained in the spermatozoon affected only the forces acting at the surface of the egg; and that the lack of this factor could be replaced by a rise

in the alkalinity of the sea water, it was to be expected that the reverse should also be possible: namely, that a change in alkalinity or the constitution of the surrounding medium should produce a reversible block to the spermatozoa of the same species. That means, it should be possible to find solutions in which the egg does not suffer for a long time, in which the sperm lives for a long time, and in which the sperm of the same species is intensely active and attacks the egg with the greatest eagerness and yet is not able to enter; while if the medium is but slightly changed the sperm enters the egg at once. The writer carried out such experiments a year ago in Pacific Grove and last summer in Woods Hole and found this to be true.⁹

For the purpose of these experiments the ovaries and testes of the sea urchins were not put into sea water but into a pure $m/2$ NaCl solution (after several washings in such a solution) and kept in such a solution. Several drops of sperm and one drop of eggs were in one experiment put into 2.5 c.c. of a neutral mixture of $m/2$ NaCl and $3/8 m$ $MgCl_2$ in the proportion in which these two salts exist in the sea water. In such a neutral solution no egg of *Arbacia* or of *purpuratus* is fertilized no matter how long it remains in the solution, although the sperm is very active. If the eggs and sperm are transferred into the same solution which contains in addition 1 drop of a $N/100$ solution of NaOH (or NH_3 , or benzylamine, or butylamine) or 8 drops of $m/100$ $NaHCO_3$, most and often practically all the eggs at once form fertilization membranes and begin to segment at the proper time.

The same result can be obtained if the eggs are transferred into a neutral mixture of $NaCl + MgCl_2 + CaCl_2$ (in the proportion in which these salts exist in the sea water) or into a neutral mixture of $NaCl + MgCl_2 + CaCl_2 + KCl$. In such a neutral mixture the eggs form fertilization membranes and begin to segment.

The eggs will not be fertilized if transferred into a neutral solution of NaCl or of $NaCl + KCl$.

It is, therefore, obvious that if we diminish the alkalinity of the solution surrounding the egg and if we deprive this solution of $CaCl_2$ we establish the same reversible block to the entrance of the

⁹ Loeb, *Science*, N. S., XL, 316, 1914.

spermatozoon of *Arbacia* into the egg of the same species as exists for the entrance of the sperm of starfish into the egg of *purpuratus* in normal sea water.

Another form of the experiment may be mentioned. When we put sperm and eggs of *Arbacia* (which had been washed in an $m/2$ NaCl solution) into a neutral mixture of NaCl + KCl no egg can be fertilized although the sperm may be so active and concentrated that the eggs roll around in the solution and the chorion (the jelly surrounding the egg) may be filled with spermatozoa. In one experiment the eggs and sperm of *Arbacia* were kept overnight in watch glasses containing 2.5 c.c. of this mixture of neutral NaCl + KCl. The next morning all the eggs were intact and not a single one was fertilized. At that time 20 drops of sea water were added to the mixture and instantly fertilization membranes were formed and practically all the eggs segmented.¹⁰

It can be shown that in this experiment the sea water added two important substances, Ca and NaOH. If NaOH alone is added to the mixture of NaCl + KCl, as a rule no egg is fertilized or only a few; if CaCl_2 is added to a neutral mixture of NaCl + KCl a number of eggs are fertilized. If both CaCl_2 and NaOH are added in the proper proportion as a rule all the eggs are fertilized.

It is perhaps important to call attention to the fact that if eggs of *Arbacia* are fertilized in sea water and if after repeated washings in a mixture of NaCl + KCl or of NaCl + MgCl_2 they are put into these solutions they will segment repeatedly in these solutions, thus showing that the eggs were really not fertilized in these two solutions in the above-mentioned experiments.

The striking fact is again that the block created by the lack of CaCl_2 or NaOH or both to the entrance of the spermatozoon is removed immediately after these substances are added. The block must be due merely to a change in the physical condition of the surface (which may be based on a rapidly reversible chemical reaction).

¹⁰ This experiment was carried out with different concentrations of sperm and it was found that only in the dishes where the concentration of sperm was sufficiently high were all the eggs fertilized upon the addition of sea water. This is perfectly natural as the majority of spermatozoa die gradually (as do also the eggs) and hence enough spermatozoa will only be alive the next day if the concentration of sperm was not too low.

In these experiments the NaCl can not be replaced by isotonic sugar solutions. The same fact was found by the writer to be true for heterogeneous hybridization.

It is of importance to call attention to the fact that the abolition of the block in the case of heterogeneous hybridization depends upon the same substances, CaCl_2 and NaOH (or some other alkali), which make normal fertilization possible. The influence of electrolytes on the fertilization of the egg of *purpuratus* by the sperm of *Asterias* is parallel to the influence of the same electrolytes on the fertilization of the same egg by the sperm of *purpuratus*; only the concentrations differ, and always in the same sense. The forces at work are, therefore, apparently the same in both cases; but we can only express surmises as to their nature. The rôle of salts as well as the rapid reversibility indicate that they are forces situated at the surface of the egg and the spermatozoon or both. In the first place we may think of surface tension conditions and in this respect it is possible that the entrance of the spermatozoon into the egg may be determined by such forces in a way similar to the process of phagocytosis. In the second place it may be that previous to the action of surface tension forces an alteration in the degree of fluidity of the egg surface may be required (*e. g.*, that physical change which finds its expression in the formation of the fertilization cone). Thirdly, it may be possible that before the surface tension forces can act the spermatozoon must agglutinate with the egg surface and that this agglutination is determined by certain specific substances or by certain salts (CaCl_2 and NaOH) or by both.

Brief mention should be made of the block discovered by Godlewski¹¹ to the entrance of a spermatozoon into the egg if the sperm of the same species is mixed with the sperm or the blood of a species widely apart. If, for instance, the sperm of a sea urchin is mixed with the sperm of certain annelids (*Chaetopterus*) or molluscs and if after some time the eggs of the same sea urchin are added to the mixture of the two kinds of sperm no egg is fertilized. If the solution is, however, subsequently diluted with sea water or if the egg that was in this mixture is washed in sea water, the same sperm mixture in which the egg previously remained unfertilized will now

¹¹ Godlewski, *Arch. f. Entwicklungsmech.*, XXXIII, 196, 1911.

fertilize the egg. From these and similar observations Herlant¹² draws the conclusion that the block existed at the surface of the egg, inasmuch as a reaction product of the two types of sperm is formed after some time which alters the surface of the egg and thereby prevents the sperm from entering. This view is not only supported by all the experiments but also by the observation of the writer that foreign sperm or blood is able to cause after some time a real agglutination if mixed with the sperm of a sea urchin or a starfish.¹³ We can imagine that the precipitate forms a film around the egg and acts as a block which can be removed mechanically by washing.

It is not impossible that the block which exists in the fertilized egg is due also to an alteration of the physical character of the surface of the egg which in this case is, however, induced from within the egg by changes caused by the entrance of the spermatozoon, which, however, are not necessarily identical with those causing development as was shown by the facts in the second chapter.

IV.

We will now turn to the question whether the motility of the spermatozoon plays no other rôle than to bring the spermatozoon so close to the surface of the egg that surface tension phenomena can engulf the spermatozoon into the egg. It is easy to show that if the spermatozoa of *purpuratus* are immobilized by NaCN no egg of the same species can be fertilized, no matter how concentrated the sperm; while the same sperm when it revives from the effect of NaCN fertilizes the same eggs at once. This meets with the possible objection that the motility of the sperm might be only necessary to allow the latter to penetrate the jelly surrounding the egg protoplasm. In order to test this objection the writer freed the eggs of *purpuratus* from this jelly by treating them for two minutes in a mixture of 50 c.c. sea water + 3 c.c. *N*/10 HCl in which all the jelly is dissolved. The eggs were washed afterwards in sea water and it was found that if sperm was added practically all were fertilized. The writer put such eggs with sperm which was immobilized by NaCN.

¹² Herlant, *Anat. Anzeiger*, XLII, 563, 1912.

¹³ Loeb, *Jour. Exper. Zool.*, XVII, 123, 1914.

The eggs and the sperm were squirted together with a pipette in order to bring about a close contact. No matter how concentrated the sperm was, not a single egg was ever fertilized. As soon as the spermatozoa recovered and showed only a slight degree of motility fertilization became possible. This leaves no doubt that the motility of the sperm is one of the forces required to bring the spermatozoon into the egg.

That motility is not the only force was already indicated by the previous chapter which made it clear that even if the sperm is active it can not enter the egg unless certain physical conditions at the phase boundaries of egg, spermatozoon and surrounding solution were right. In order to leave no doubt about this fact the following experiments may be quoted. If we put NaCl sperm¹⁴ of *purpuratus* or of *Arbacia* into a neutral mixture of NaCl + KCl containing eggs of the same species the sperm will sooner or later become very active. Yet not a single egg is fertilized. If we make the solution slightly alkaline the sperm becomes at once extremely active yet with a few exceptions no egg is fertilized; while much less active sperm will fertilize all the eggs if CaCl₂ is added. The second fact is this: that the most active sperm of *Asterias* will not fertilize the eggs of *purpuratus* in sea water while it will do so in hyperalkaline sea water (50 c.c. sea water + 0.6 c.c. N/10 NaOH).

We, therefore, arrive at the conclusion that aside from the physical conditions at the surface of the egg and the spermatozoon the impact of the spermatozoon against the egg is a prerequisite for the process of fertilization.

Von Dungern was, as far as the writer is aware, the first to call attention to the fact that the egg itself causes resting spermatozoa to become active,¹⁵ but curiously enough he tried to show that only foreign sperm is "stimulated" in this way by the egg (which is, as F. Lillie pointed out, not correct) and v. Dungern tried to explain on this basis why it was not possible to fertilize the egg of the sea urchin with the sperm of the starfish which had at that time not yet been accomplished.

Von Dungern noticed that the egg of the sea urchin "stimulates"

¹⁴ Sperm from testicles washed in $m/2$ NaCl and kept in such a solution.

¹⁵ v. Dungern, *Ztsch. f. allg. Physiol.*, I, 34, 1902.

the spermatozoon of starfish to greater action and he concluded that since "stimulation" according to Jennings causes "motor reaction" whereby the direction of the motile organism is changed this very stimulating influence of the egg of the sea urchin upon the spermatozoon of the starfish prohibited the latter from getting into the egg. On the basis of the same idea von Dungern was consistently led to the further conclusion that the egg exercised no "stimulating" influence upon spermatozoa of its own species and that thereby the spermatozoon of the same species was enabled to get into the egg. A year after the appearance of von Dungern's paper the writer succeeded in accomplishing the hybridization of the sea urchin egg with starfish sperm by a method which contradicted von Dungern's theory, namely, by increasing the alkalinity of the sea water whereby the spermatozoon is "stimulated" to still greater activity; and on the other hand it is a common experience that a sea urchin spermatozoon becomes more active when it comes near an egg of its own species.

The writer was anxious to compare the activating action of eggs of the same and various foreign species upon spermatozoa. Since the spermatozoa of the sea urchins are usually very active in pure sea water (*i. e.*, sea water free from egg substance) it was necessary to find a solution in which these spermatozoa will keep alive for a number of days without showing any motility. Such a solution was found in a neutral $m/2$ NaCl solution and this led to the method of putting ovaries and testes directly into such solutions instead of into sea water.¹⁶ The ovaries and testes were first washed repeatedly in these solutions to free them from the blood or its salts, and then one drop of eggs and one or more drops of the sperm suspension were mixed in a watch glass containing 5 c.c. $m/2$ NaCl (free from egg contents). In one experiment the sperm and eggs of two sea urchins, *purpuratus* and *franciscanus*, and two starfish, *Asterias ochracea* and *Asterina* (at Pacific Grove), were used. None of the

¹⁶ The writer had found previously that the unfertilized eggs of *purpuratus* are killed more rapidly in sea water than in a neutral $m/2$ NaCl solution, probably on account of the greater alkalinity of the former. The same may be true for the sperm of this species, although this has not yet been tested. The unfertilized egg of *Arbacia* is more sensitive to a pure NaCl solution than that of *purpuratus*.

four forms of spermatozoa showed any motility in a pure NaCl solution (without egg contents). In sea water (free from egg contents) the spermatozoa of the two forms of sea urchins were very active, those of the starfish were immobile. The starfish eggs were immature and did not mature during the experiment (those of *Asterias* were out of season and very small); the sea urchin eggs were mature. The result is indicated in the following table.

TABLE I.
Specificity of Activation of Sperm by Eggs.

	<i>Asterias</i> ♂	<i>Asterina</i> ♂	<i>Franciscanus</i> ♂	<i>Purpuratus</i> ♂
<i>Asterias</i> ♀ (immature)..	<i>Immediately very motile.</i>	No activation.	Moderately active.	Slight effect in immediate contact with egg.
<i>Asterina</i> ♀ (immature).	Not motile.	<i>Violent activity.</i>	<i>Violent activity.</i>	Slight effect only near the egg.
<i>Franciscanus</i> ♀ (mature)	Slightly motile.	No motility.	<i>Immediately active.</i>	<i>Immediately motile.</i>
<i>Purpuratus</i> ♀ (mature).	Slightly motile after some time.	Slight effect in immediate contact with eggs.	<i>Immediately active.</i>	<i>Immediately active.</i>

That there exists no strict specificity is obvious by the fact that the immature eggs of *Asterina* activate the sperm of the sea urchin *franciscanus* as powerfully as is done by the mature eggs of the sea urchin *purpuratus* and *franciscanus*. But the spermatozoa of the two species of starfish show a marked specificity inasmuch as they are activated strongly only by the (immature) eggs of their own species and only to a slight degree by the eggs of the sea urchin *purpuratus*. In judging these results the reader must keep in mind first that all these experiments are made in a NaCl solution, and second, that it requires a stronger influence to activate the spermatozoa of the starfish which are at first not motile in sea water (free from egg contents) than the sea urchin spermatozoa which are from the very first very active in such sea water and which may therefore be considered as being at the threshold of activity in the pure NaCl solution.

If instead of the eggs themselves the supernatant NaCl solution from eggs is added to the sperm it is found that it requires a very much greater concentration of the supernatant NaCl solution from

Asterias eggs to arouse the *purpuratus* sperm in NaCl into activity than if the supernatant NaCl solution from *purpuratus* or from *franciscanus* eggs is used.

The question now arises whether the relative influence of the egg on the motility of the sperm bears any relation to the power of the latter to enter the egg; or in other words if we can foretell which forms will hybridize by observing the relative activating effect of the eggs upon the spermatozoa. This does not appear to be the case on the basis of our present limited experience, since the activating effect of the *franciscanus* egg upon the sperm of *Asterias* is just as great as if not greater than that of *purpuratus* eggs and yet *Asterias* sperm can enter the latter and not the former. Even if we intensify the activity of the spermatozoon of *Asterias* by putting it in hyperalkaline sea water it will not enter the egg of *franciscanus*.

If we mix eggs of *franciscanus* and *purpuratus* in sea water and add the sperm of *purpuratus* the eggs of *purpuratus* will be fertilized more quickly than the eggs of *franciscanus*; and the reverse is true if the sperm of *franciscanus* is added to a mixture of both eggs in sea water. The writer is not quite certain that this difference is accompanied by a corresponding difference in the influence of these eggs upon the motility of their spermatozoa. It is certain, however, that the addition of egg sea water from *Asterias* does not help the fertilization of *purpuratus* eggs by *Asterias* sperm, although the egg sea water from *Asterias* increases the activity of *Asterias* sperm.

The writer is, however, of the opinion that this activating effect of the egg upon the spermatozoon is of the greatest importance for fertilization in nature and that the degree of specificity which exists (although it is far from absolute) is a means of preventing hybridization. The writer is under the impression that the eggs which are naturally fertilized in water are fertilized almost instantly after they are shed. Thus it is stated at hatcheries that the egg of the salmon loses its power of being fertilized in a few minutes and in the case of *Fundulus* the egg loses this power also very rapidly. The ripe egg of starfish dies rapidly if not fertilized. On the other hand, the writer has often been struck with the fact that the sperm of most marine forms when put into sea water is at first practically not motile. When the eggs have a specific gravity considerably greater

than the water (as is the case for *Fundulus*) the eggs will sink very rapidly while the sperm remains suspended for some time. Now we have mentioned that if the absolutely inactive sperm of *Asterias* or *Asterina* comes in contact with eggs of its own species (even if they are immature) it is at once aroused into violent activity. If the same were true for the egg of *Fundulus* fertilization could take place probably before the egg reaches the bottom of the water. If by chance a teleost of a different species would shed its sperm in the immediate neighborhood and some of it could reach the egg of *Fundulus* while it is falling the foreign sperm could probably not be aroused as quickly by the egg of *Fundulus* as the sperm of the *Fundulus* male and hence no hybridization would occur. In fish we can see that the male and female shed their sexual cells simultaneously so that they come at once in contact. The writer is inclined to believe that something similar occurs also in Echinoderms. He had last year a chance to verify once more an observation he had made for a number of years and which he had already mentioned in a previous publication.¹⁷ The sea urchins at Pacific Grove are found in large numbers on rocks in certain coves near the shore. Up to a certain day in March every female of *purpuratus* was full of eggs. On the next day the surface of the sea in this region showed the usual indication of the spawning of large masses of animals: namely the enormous foam formation in the little coves although the sea was only moderately agitated. This foam formation is due to an increase of organic substances which lower the surface tension of the sea water and make the foam more durable. The writer realized that this might mean the end of material for some time to come and indeed not a single female of *purpuratus* of hundreds opened on that day had eggs. The condition was the same for all the sea urchins collected for two miles along the shore. During the next week immature eggs began to appear again in the sea urchins and in about ten days ripe eggs were again found. This indicates that in this region the males and females shed their eggs and sperm simultaneously. It is not impossible that among sea urchins which are found in colonies on the rocks the shedding of the sexual products of one or several individuals acts as an incentive

¹⁷ Loeb, "The Mechanistic Conception of Life," Chicago, 1912, p. 196.

for the whole colony. Since the eggs fall in this case also much more rapidly to the bottom than the spermatozoa it is also very probable that the eggs are fertilized before they reach the bottom of the sea. We can understand under these circumstances that the specificity which exists in the activating effect of the egg upon the sperm is one of the safeguards against hybridization for eggs that are fertilized in the water, inasmuch as this specificity activates the sperm of the same species much more quickly than that of a foreign species. Other safeguards are the phase-boundary conditions which we discussed in the previous chapter.

V.

If we assume that the spermatozoon bores itself into the egg by the energy of the vibrations of its flagellum it is easy to understand the importance of its motility for this process. It is, however, equally possible that a certain energy of vibration is needed to make the spermatozoon stick to the surface of the egg and that afterwards forces of a different character bring the spermatozoon into the egg. The fact that under normal conditions a very slight degree of motility on the part of the spermatozoon allows it to enter the egg seems to favor such a view.

Von Dungern had already discussed the possible rôle of phenomena of sperm agglutination in fertilization as a protective agency. F. Lillie discovered the transitory agglutination of sperm induced by a substance from eggs of the same species.¹⁸ When the sperm of the sea urchin *Arbacia* is mixed with the supernatant sea water from eggs of the same species a cluster formation occurs which may last a number of minutes and which is essentially a transitory agglutination. In *Arbacia* the agglutination is very striking, in *purpuratus* the phenomena of agglutination are not lacking but the writer was under the impression that other phenomena of the type of tropisms might enter. But he was not very certain on this point and left that question open for further discussion. The writer is, however, under the impression that no proof for the existence of a positive chemotropism of the sea urchin sperm for the eggs of the same species has thus far been given.

¹⁸ F. Lillie, *Science*, N. S., XXXVIII, 524, 1913; *Jour. Exper. Zool.*, XVI, 523, 1914.

The writer observed that this phenomenon of sperm agglutination depends on the motility of the sperm:¹⁹ It only appears when the sperm is extremely motile and it lasts only a number of minutes, often only a fraction of a minute as Lillie had found. The writer observed that the duration of the clusters depended to some extent on the alkalinity of the solution. The more alkaline the latter the more rapidly the cluster scatters. The presence of a salt with a bivalent metal, especially Ca, seems necessary for the cluster formation. Sr and Ba act like Ca and so does Mg but in the latter case a slightly higher concentration is needed. The more Ca is added the more powerful the agglutination becomes. These facts suggest the following origin of the agglutination. From the jelly surrounding the eggs a certain substance is dissolved in the sea water which reacts chemically with a certain substance at the surface of the spermatozoon. If this reaction takes place in the presence of one of the salts of a bivalent metal, especially Ca, a sticky precipitate is formed on the surface of the spermatozoa, which is slowly soluble in the solution; and the more rapidly the more alkaline the solution. If the spermatozoa are very active the impact with which they strike each other may lead to their sticking together and this agglutination will last until the precipitate is dissolved again.²⁰

The writer mentions this fact here because it might give us a clue to the rôle of the motility of the spermatozoon for its entrance into the egg. One can imagine that the spermatozoon must stick to the surface of the egg in order to be taken into it and this sticking may not come about unless the spermatozoon strikes the surface of the egg with a certain velocity. This is, however, merely a suggestion. The really serious difficulty of such an assumption lies in the fact that the specific and transitory cluster formation or agglutination of the spermatozoa is not a general phenomenon. It may even turn out to be confined to sea urchins and certain annelids. It is probably lacking in all cases of hybridization. Yet this would not necessarily speak against the possibility of an agglutination of the spermatozoon to the egg as a prerequisite of fertilization.

¹⁹ Loeb, *Jour. Exper. Zool.*, XVII, 123, 1914.

²⁰ Lillie measures the degree of agglutination by its duration; if our assumption is correct he really measures the time required for the solution of the sticky precipitate on the surface of the spermatozoon by the sea water.

This latter idea receives some support in the writer's experiments on heterogeneous hybridization. He was able to show that both NaOH as well as CaCl_2 , which render possible the fertilization of the eggs of certain sea urchins through the sperm of starfish, also favor the agglutination of that sperm to the chorion of the egg. This leads to the peculiar phenomenon of mere membrane formation in the egg by the living spermatozoon without the entrance of the latter into the egg.²¹

VI.

Lillie seems to take it for granted that the substance of the egg which causes sperm agglutination is identical with the substance which stimulates the spermatozoa into greater activity. If this were correct the conditions for the two phenomena should be identical, which is however far from being the case.

The writer showed that if we deprive the eggs of *purpuratus* of the jelly which surrounds them and if we wash them afterwards a few times in sea water to deprive them of the last vestiges of jelly substance which may still adhere to them they have lost completely and permanently the power of forming clusters with the sperm of their own species. Such eggs were washed four times in $m/2$ NaCl and when a drop of the supernatant NaCl solution was added to NaCl sperm of *purpuratus* which was not motile it activated the sperm very powerfully.

The writer had found that the egg sea water of *S. franciscanus* does not give a trace of agglutination with the sperm of *purpuratus* but if the experiment is made in $m/2$ NaCl solutions it can be shown that the *franciscanus* egg NaCl solution activates the NaCl sperm of *purpuratus* in an $m/2$ NaCl solution very strikingly.

The immature eggs of *Asterias ochracea* activate the otherwise non-motile sperm of the same species, but the eggs of this starfish do not give any agglutination reaction with their own sperm and Lillie found the same for the starfish in Woods Hole. It might be said that all this only proves that the activating effect requires a smaller concentration than the agglutinating effect, but may yet be caused by the same substance. This objection is, however, not tenable in the following case.

²¹ Loeb, *Arch. f. Entwicklungsmech.*, XL, 310, 1914.

Purpuratus sperm washed in $m/2$ NaCl is as a rule more active in a mixture of NaCl + KCl than in a mixture of NaCl + CaCl_2 (if both solutions are free from egg contents); yet in the latter solution the agglutination reaction upon the addition of egg NaCl is very strong while in the former it is lacking (unless the sperm or testicles or ovaries give off some CaCl_2 to the surrounding solution). Again it might be argued that the activation of the spermatozoon might be induced by the same substance as the agglutination, but that the agglutinating substance in both cases reacted with different constituents of the spermatozoon. While this may be admitted, it must also be conceded that with the facts which we have at our disposal at present we can not be certain that the agglutinating and activating substances are identical.

VII.

Lillie²² not only takes the identity of the two substances for granted but he assumes that without the agglutinating substance in the egg (to which he gives the somewhat prejudicial name "fertilizin") no fertilization is possible. Fertilization in his opinion consists in the combination of the spermatozoon with a molecule of "fertilizin" in the egg, whereby the fertilizin molecule undergoes a change in the other end and this change causes the egg to develop. The fertilizin is thus an "amboceptor" in the sense of Ehrlich's side-chain theory.

The side-chain theory was invented by Ehrlich for an altogether different purpose. Bordet had found that for certain phenomena of immunity two substances were needed (which Ehrlich named amboceptor and complement, respectively). Ehrlich assumed that they were bound chemically by the antigen (the substance against which the organism was immunized) but found that while the antigen (A) was able to bind B (the amboceptor) in the absence of C , it was not able to bind the complement C in the absence of B . From this Ehrlich concluded that of the two possible modes of

linkage between the three bodies $A \begin{matrix} \swarrow B \\ \searrow C \end{matrix}$ and $A-B-C$ the latter

²² *Loc. cit.*

was the one which really occurred. Since in this case *C* is not directly linked with *A* but through the intermediation of *B* he called *B* the "amboceptor" and the scheme of linkage a "side-chain" linkage.

Lillie applies this theory (which covers the two possible modes of linkage of two chemical compounds to a third one) to the entrance of the spermatozoon into the egg, by calling the egg an antigen *A* and the spermatozoon a complement *C* and assuming the existence of a hypothetical amboceptor *B* in the form of the substance that causes agglutination, the "fertilizin." Even if we are willing to overlook the fact that the egg and the spermatozoon are cells and not simple organic compounds and if we are willing to overlook the further fact that the assumption of an amboceptor as a connecting link between the two is arbitrary we can not overlook the fact that the spermatozoon does not combine chemically with the egg but that it actually enters into the egg and attaches itself to the egg nucleus. It seems then futile to discuss whether the spermatozoon combines with the egg in side-chain fashion (namely, Egg—Fertilizin—Spermatozoon) or in direct fashion, namely,



since the engulfing of the spermatozoon into the egg is a physical process which bears no relation to either possibility.

It has been stated that the "fertilizin theory" explains also the phenomena of artificial parthenogenesis just as well as any other theory. In a recent book on artificial parthenogenesis the writer has given the results of a large number of experiments and he has tried to explain some of them; the reader would, however, vainly look for a "theory" of artificial parthenogenesis. A theory in a scientific sense consists in the presentation in mathematical or numerical form of a phenomenon as the function of its variables. The writer has tried to prepare the ground for such a treatment of the phenomena of fertilization and of the first development of the egg by working out those variables which permit a quantitative treatment, but even if the exploration had been advanced further than it actually has been, it would not be possible to ever expect that a single theory

could cover all the phenomena of fertilization and development, since under these two headings so many physically and chemically different processes are included (of which one follows the other) that they can not be covered by one theory. It is true the writer had in former publications occasionally used the term "lysin theory of fertilization" but only to express the fact that cytolytic agencies induce membrane formation and that the membrane formation induced by a spermatozoon might also be due to a cytolytic agency contained in the spermatozoon; but he has dropped this term in his recent book on the subject.

While the writer does not desire to enter into a further discussion of the side-chain theory of fertilization he wishes to point out that it rests on the claim that that substance which causes sperm agglutination is contained in the unfertilized egg and that the egg can only be fertilized as long as this "fertilizin" is present in the egg. It is obvious that such an assumption demands for its proof that in all cases in which an egg can be fertilized it must contain the agglutinating substance. There is only one test for the presence of this substance, namely the cluster formation of the sperm in the presence of egg sea water. This proof can not be furnished since, as the writer had shown in a former paper, the reaction is lacking in many cases of hybridization; it is also lacking in the case of the starfish.²³ It is not impossible that if the theory is tested further it will be found lacking in a considerable number of cases. To this objection Lillie replies that it is not necessary that the eggs should actually give the agglutinin reaction, it is sufficient that the agglutinating substance is contained in the egg. But how can we tell that it is contained in an egg which fails to give the agglutination reaction as long as this reaction is the only reliable test for the presence of the agglutinating substance in the egg? Rigorously speaking, even if all eggs of every species gave the agglutinin reaction it would still be necessary to furnish a direct proof that the agglutinin has anything to do with fertilization and development.

It may be possible that Lillie considers such a proof to be contained in the following statement.

²³ Lillie, *Biol. Bull.*, XXVIII, 18, 1915.

I adopted then the working hypothesis that this substance²⁴ is necessary for fertilization and there followed immediately three corollaries, viz.: (1) if it were possible to extract this substance from eggs they would no longer be capable of fertilization; (2) fertilized eggs are incapable of uniting again with spermatozoa, hence if the hypothesis is correct they could no longer contain free fertilizin; (3) eggs in which membranes have been formed by methods of artificial parthenogenesis become incapable of fertilization; such eggs must also therefore be devoid of free fertilizin after they have reached the non-fertilizable condition if the hypothesis is correct. These consequences were actually found to be true.²⁵

Of these three "corollaries" the first one is the most important, since it claims that the power of the eggs of being fertilized varies with their contents of fertilizin. The proof consisted in this: that eggs were washed a number of times during three consecutive days and after two days the percentage of eggs that could be fertilized were diminished to about one third.

There is thus the anticipated decrease in the percentage of fertilizations.

It is a well-known fact that the unfertilized eggs of the sea urchin (in fact of all marine animals) perish when they lie for some time in sea water and one of the main causes of this phenomenon is also known, namely oxidations. If the oxidations are inhibited through the removal of oxygen or the addition of KCN the life of the eggs can be prolonged.²⁶ In the mature starfish egg this death which is accelerated by the temperature (and has the high temperature coefficient of many life phenomena) takes place in a few hours,²⁷ while it begins a little later in the egg of the sea urchin. After the artificial membrane formation it takes place very rapidly also in the sea urchin egg (coincident with the enormous increase in the rate of oxidations caused by the artificial membrane formation) and in this case the death of the egg can also be retarded by the withdrawal of oxygen or the addition of cyanide.²⁸ In view of these facts the objection can not be avoided that in Lillie's experiment the number of eggs which could be fertilized fell off after two days to one third not on account of the loss of "fertilizin" but because of the fact

²⁴ The "fertilizin."

²⁵ Lillie, *Jour. Exper. Zool.*, XVI, 523, 1914.

²⁶ Loeb and Lewis, *Am. Jour. Physiol.*, VI, 305, 1902.

²⁷ Loeb, *Biol. Bull.*, III, 295, 1902.

²⁸ Loeb, "Artificial Parthenogenesis and Fertilization."

that two thirds of the eggs were dead by that time. That this assumption is well grounded is testified by Lillie's own remarks:

Concomitantly, with these effects of the series of washings, the developmental energy becomes greatly reduced. This was very obvious from the second fertilization.²⁹ On August 24 (48 hours after fertilization) a large quantity of living material was contained in the second *A* fertilization but none had even approximately pluteus structure. The most common form was a stereoblastula. In the second *B* fertilization there were a few abnormal prismatic plutei, while the majority was gastrulae. The third fertilization resulted in extremely abnormal ciliated types. The fourth and fifth did not proceed beyond abnormal cleavage stages.

From this and similar experiments Lillie draws the following conclusion:

The eggs have evidently lost something which affects their power of fertilization. Table 3 shows the measure of loss of the sperm agglutinating substance and justifies the general conclusion that this is a factor in the result. The loss of other substances may also combine in the decrease of fertilizing power, but of this we know nothing definite. As a matter of fact, fertilizing power is gradually lost with decrease of fertilizin content of the egg.

It seems to the writer that in these experiments the power of being fertilized was gradually lost by the death of the eggs. And an additional justification of this criticism is given by the following fact, that if we deprive *fresh* eggs of *purpuratus* permanently of their power of giving off "fertilizin" their power of being fertilized is not only not lost but is entirely unaltered. The writer has shown that if the eggs of *purpuratus* are treated for two or three minutes with a mixture of 50 c.c. of sea water + 3 c.c. of HCl (whereby the jelly surrounding the egg is dissolved) and if the eggs are washed they give no trace of a fertilizin reaction but 100 per cent. of the eggs can be fertilized.³⁰

It might be argued that the supernatant sea water from these eggs had not lost all power of causing agglutination of the sperm. This the writer must deny but for arguments' sake he will admit that a trace near the "psychological limit" might have been overlooked where a "fertilizin" partisan might have declared that he still could perceive a faint indication of a "fertilizin" reaction. In that case

²⁹ Which occurred on the second day.

³⁰ Loeb, *Jour. Exper. Zool.*, XVII, 123, 1914.

only a few eggs should have been fertilized—the fertilizin theory rests on this assumption; in reality, however, practically one hundred per cent. were fertilized in every case (provided the eggs had not been lying in the sea water too long, *i. e.*, more than a day or two).

To this Lillie replies that perhaps the sperm of *purpuratus* is not so delicate an indicator for agglutinin as the sperm of *Arbacia*—but as long as the agglutination reaction is the only test for the presence of fertilizin in the egg, such an answer begs the question.

From the fact that the power of agglutinating the sperm is lost if the egg of *purpuratus* is deprived of its jelly by acid treatment the writer drew the conclusion that in this egg the “fertilizin” does not come from the unfertilized egg but only from its jelly and that this was contrary to Lillie’s assumption. To this Lillie⁸¹ replied by pointing out that the immature eggs of *Arbacia* do not give the agglutination reaction while the mature *Arbacia* egg gives the reaction very powerfully, and that we must conclude from this that the “fertilizin” contained in the jelly comes from the egg and is given off during the period of the maturation divisions (the latter statement, however, is after all only an assumption though a probable one). But this does not meet the question at issue, namely that in the egg of *purpuratus* at the time of maturity the fertilizin which is given off is contained exclusively in the jelly and not in the egg, as it should be if the presence of fertilizin in the egg were a prerequisite for its ability of being fertilized. It is true that if we repeat this experiment in the egg of *Arbacia* we find that after the removal of the jelly by HCl a trace of the agglutinating substance may still be given off by the egg, although little in comparison with that given off by the jelly. But this does not alter the facts as they are found in the egg of *purpuratus*.

As far as the two other proofs of Lillie are concerned, we have already touched upon them in the previous parts of this paper. The fact that the fertilized eggs of *Arbacia* (and of *purpuratus*) cease to give the agglutinin reaction is due to the loss of the jelly on the part of the fertilized egg to which in *Arbacia* should be added the fact that some of the material of the cortical layer is given off during the process of membrane formation. The writer has pointed out in

⁸¹ Lillie, *Biol. Bull.*, XXVIII, 18, 1915.

former papers that the cortical layer of the egg which undergoes liquefaction in the process of membrane formation behaves towards reagents very much like the jelly which surrounds the egg.³² But since in the egg of *purpuratus* the loss of this agglutinating power on the part of the egg is not necessarily accompanied by the loss of the power of being fertilized—*e. g.*, in the HCl experiment—we are inclined to believe that there must be another reason that an egg fertilized by sperm can not be fertilized a second time.

As far as the statement is concerned that the egg can no longer be fertilized after artificial membrane formation by butyric acid the writer can not admit the correctness of this statement (see Chapter III). In the eggs in which artificial membrane formation has been called forth by butyric acid the main if not the only block to a subsequent fertilization is the membrane itself.

This can be proved by a very simple experiment. If we call forth the membrane formation in the egg of *purpuratus* in a neutral or faintly alkaline solution of $m/2$ ($\text{NaCl} + \text{KCl} + \text{CaCl}_2$) (instead of in sea water) a very thin membrane is formed, which is easily torn and offers no resistance to the spermatozoon. All the eggs treated in this way can be fertilized by sperm. The agglutinin reaction of such eggs is, however, permanently lost.

The facts thus far known seem to force us to the conclusion that no adequate proof has been offered thus far for the connection between the power of an egg of being fertilized by sperm and its power of causing a cluster formation of the sperm. The writer has pointed out in a previous paper that it is difficult to see why there should exist such a relation, since sperm agglutination can only inhibit the entrance of the spermatozoon into the egg.

³² "Artificial Parthenogenesis and Fertilization," Chicago, 1913, pp. 210-14. University of California publication, Physiology, Vol. 3, p. 1, 1905.

FURTHER EXPERIMENTS ON THE RELATIVE EFFECT OF WEAK AND STRONG BASES ON THE RATE OF OXIDATIONS IN THE EGG OF THE SEA URCHIN.

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When Warburg discovered that NaOH raises the rate of the oxidations in the fertilized eggs of the sea urchin, although it does not enter the egg,¹ he made an important addition to our knowledge of the localization of oxidations in the cell. He correctly concluded that this leaves no doubt that the oxidations raised by NaOH must be located at the surface of the egg. If we are prepared to admit this, we are compelled to face the possibility that the interior of the cell receives little or no oxygen, since whatever oxygen may diffuse to the egg will be chiefly, if not all, used up at the surface of the cells.

In Warburg's observations the weak base NH_4OH had no influence on the rate of the oxidations in the fertilized egg, although it enters the latter. This would be intelligible on the assumption that no oxidations or practically none occur inside the cell, and that the oxidations at the surface of the cell are accelerated by the hydroxyl ion and not by the molecule of the base. There are, however, facts which are not in harmony with both of these conclusions. Unfertilized eggs of the sea urchin as well as of annelids can be induced to develop under the influence of bases, and the bases are only active in the presence of oxygen. The activating action of the bases is also inhibited by the addition of KCN or NaCN.² The activation of the egg consists in a change of the surface layer of the egg (which

¹ O. Warburg: *Ztschr. f. physiol. Chem.*, lxvi, p. 305, 1910.

² J. Loeb: *Artificial Parthenogenesis and Fertilization*, Chicago, 1913, p. 147 and ff.

may or may not result in a membrane formation) and the bases bring about this change by accelerating or starting certain oxidations in the surface layer or at the surface of the eggs. The writer showed that for this change the weaker bases NH_4OH and the amines are equally, if not more efficient than the strong bases NaOH or tetraethylammoniumhydroxide. We must, therefore, conclude either that in this case the bases accelerate oxidations not through the OH ion but by the influence of the molecule, or that, if the effect of bases on oxidations is due to the OH ion, oxidations cannot be confined to the surface of the egg but must take place also inside the egg or at least in the cortical layers adjacent to the surface of the egg.

It was found also that the maximum effect of the weaker bases was reached at a comparatively low concentration, while that of the strong bases increases steadily with the concentration.³ This could speak in favor of either possibility. We felt that further experiments would be required to clear up this ambiguity. We chose for our purpose the fertilized egg instead of the unfertilized, since in the latter the objection may be raised that the increase of the rate of oxidations by bases is merely an indirect one and due to the activation of the egg by the base. We selected the egg of *Arbacia*.

Method and Results.

Eggs of *Arbacia* were fertilized in sea water, and then washed three times or more in a neutral mixture of $\frac{M}{2}$ ($\text{NaCl} + \text{KCl} + \text{CaCl}_2$) in the proportion in which these salts occur in the sea water. Next a homogeneous suspension of them was made. This suspension was divided into several equal parts. One part remained in the neutral $\frac{M}{2}$ ($\text{NaCl} + \text{KCl} + \text{CaCl}_2$) solution; to the other were added varying amounts of NaOH or NH_4OH , and the rate of oxidations for one hour was determined by Winkler's method. The percentage increase of the rate of oxidations of the eggs in the alkali over those in the neutral solution was thus ascertained.

The following two tables give the results of the experiments, and the results are expressed graphically in the two curves of Fig. 1. In this figure the abscissae give the amount of cc. of $\frac{N}{10}$ base added

³ J. Loeb and H. Wasteneys: this *Journal*, xiv, p. 355, 1913.

to 100 cc. of $\frac{M}{2}$ ($\text{NaCl} + \text{KCl} + \text{CaCl}_2$), and the ordinates represent the rise in the rate of oxidations above that at the neutral point. Two facts are salient from these data; namely, first, that the effect of NH_4OH is considerably greater than we should expect if both NaOH and NH_4OH acted alike (*e.g.*, only at the surface of the egg) and if both acted merely through their OH ion concentration; and second, that the curves of the effects of both run closely parallel. The falling off of the rate of oxidations if the concentration of the base exceeds a certain limit may be accounted for by a secondary effect and may remain outside of the discussion.

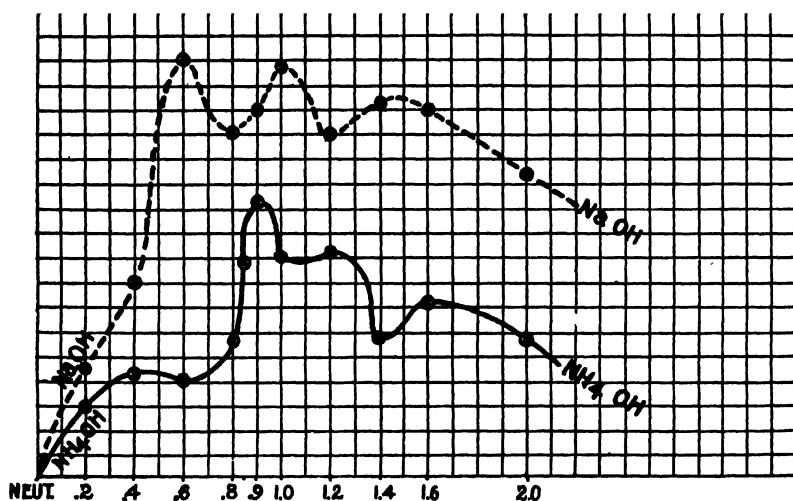


FIG. 1.

The fact that NaOH acts more powerfully than NH_4OH excludes the idea that we are dealing here with an effect of the undissociated molecule; and the parallelism in the effects of the two bases suggests a common source of their action. Such a common source could exist only in the OH ion (if the action of the molecule is excluded). The fact that the weak base acts more powerfully than we should expect from its degree of dissociation indicates that this effect is overcompensated by an advantage which the weak base has. This may be the fact that the weak base diffuses into the egg while

the strong base does not.⁴ In this case one can explain all the phenomena on the assumption that while the greater part of oxygen is consumed at the surface of the egg, some diffuses into the egg and is consumed in the layers adjacent to the surface; and the more so the nearer the surface these layers are. While the weak base can accelerate the rate of the oxidations both at the surface and beneath the surface layer the strong bases are confined in their action to the

TABLE I.

Increase in the Rate of Oxidations of the Fertilized Egg of Arbacia through the Influence of NaOH. Temperature 20° C.

NO. OF EXPERIMENT	NATURE OF SOLUTION	OXYGEN CONSUMED	COEFFICIENT OF OXIDATIONS
		mg.	
I	Neutral M/2 (NaCl + KCl + CaCl ₂)	0.75	1.00
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 1.0 cc. N/10 NaOH	2.01	2.68
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 1.6 cc. N/10 NaOH	1.87	2.49
II	Neutral M/2 (NaCl + KCl + CaCl ₂)	0.75	1.00
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 0.2 cc. N/10 NaOH	1.08	1.44
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 0.8 cc. N/10 NaOH	1.82	2.42
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 1.4 cc. N/10 NaOH	1.90	2.53
III	Neutral M/2 (NaCl + KCl + CaCl ₂)	0.51	1.00
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 2.0 cc. N/10 NaOH	1.14	2.24
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 0.9 cc. N/10 NaOH	1.28	2.50
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 0.6 cc. N/10 NaOH	1.29	2.53
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 1.2 cc. N/10 NaOH	1.15	2.25
IV	Neutral M/2 (NaCl + KCl + CaCl ₂)	0.47	1.00
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 0.4 cc. N/10 NaOH	0.85	1.80

surface. This would account for the relatively excessive efficiency of the weak base.

We must then still explain the discrepancy between Warburg's observation and ours. Warburg found no effect of NH₄OH on the rate of oxidation of the fertilized egg, but this can be accounted

⁴ We have thought also of the possibility of a specific effect of the weak base, that would presuppose the action of the undissociated molecule (which we have discarded) or of the kation; but since we know of the effect of the OH ion, the assumption of an effect of the kation to the exclusion of the OH ion seemed rather forced.

for by the fact that he worked on the egg of a different species. Our results in the egg of *purpuratus* differ also from those in *Arbacia*. In the fertilized egg of *purpuratus* the NaOH acted only when it was used in a very high concentration ($> 10^{-3}$ N), in which

TABLE II.

Increase in the Rate of Oxidations of the Fertilized Egg of Arbacia through the Influence of NH₄OH. Temperature 20° C.

NO. OF EXPERIMENT	NATURE OF SOLUTION	OXYGEN CONSUMED	COEFFICIENT OF OXIDATIONS
		mg.	
I	Neutral M/2 (NaCl + KCl + CaCl ₂)	0.51	1.00
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 0.6 cc. N/10 NH ₄ OH . . .	0.72	1.41
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 1.2 cc. N/10 NH ₄ OH . . .	0.98	1.92
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 1.4 cc. N/10 NH ₄ OH . . .	0.81	1.59
II	Neutral M/2 (NaCl + KCl + CaCl ₂)	0.64	1.00
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 0.8 cc. N/10 NH ₄ OH . . .	1.01	1.58
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 1.0 cc. N/10 NH ₄ OH . . .	1.22	1.91
III	Neutral M/2 (NaCl + KCl + CaCl ₂)	0.32	1.00
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 1.6 cc. N/10 NH ₄ OH . . .	0.55	1.72
IV	Neutral M/2 (NaCl + KCl + CaCl ₂)	0.68	1.00
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 0.4 cc. N/10 NH ₄ OH . . .	0.98	1.44
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 0.9 cc. N/10 NH ₄ OH . . .	1.44	2.12
V	Neutral M/2 (NaCl + KCl + CaCl ₂)	0.73	1.00
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 0.2 cc. N/10 NH ₄ OH . . .	0.95	1.30
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 0.85 cc. N/10 NH ₄ OH . . .	1.38	1.89
VI	Neutral M/2 (NaCl + KCl + CaCl ₂)	0.51	1.00
	100 cc. M/2 (NaCl + KCl + CaCl ₂) + 2.0 cc. N/10 NH ₄ OH . . .	0.80	1.57

it must have had an etching effect on the surface,⁵ while in the egg of *Arbacia* it acted in a much lower concentration. We are not in a position to account for these specific differences in different eggs.

If our conclusion is correct it compels us to consider not only the surface but also the layers adjacent to the surface of a cell as the seat of oxidations. According to this view the greater rate of oxidations at the surface would be due to the fact that the greater part of the available oxygen is used up here so that comparatively little can diffuse into the cell.

⁵ Loeb and Wasteneys: *loc. cit.*, p. 459.

ON THE INFLUENCE OF BALANCED AND NON- BALANCED SALT SOLUTIONS UPON THE OSMOTIC PRESSURE OF THE BODY LIQUIDS OF FUNDULUS.

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I.

In former publications Loeb has described a method which offered an indirect proof for his assumption that a pure NaCl solution of a sufficiently high concentration rendered the cell membranes permeable, while the addition of CaCl₂ counteracted this destructive effect of a pure solution of NaCl. This method consisted in measuring the time during which the fertilized eggs of *Fundulus* were able to float on solutions of a higher specific gravity than the egg. Thus it was found that the egg will float on a mixture of 100 cc. 3 M NaCl + 1 cc. 10/8 M CaCl₂ for several days, during which time the embryo will continue to live and develop; while in a pure 3 M solution of NaCl the embryo will die and the egg will sink to the bottom of the test-tube in a few hours; and in a 10/8 M solution of CaCl₂ it will sink in less than one-half hour.¹ Loeb assumes that in the mixture of NaCl + CaCl₂ the embryo can live and the egg can continue to float, since the membrane remains intact for several days; for it can be shown that a mixture of 3 M NaCl + 1 cc. CaCl₂ kills the embryo of the young fish almost instantly after hatching. The embryo can, therefore, remain alive in such a solution because the membrane remains impermeable for the solution, while each of the constituents alone at this high concentration rapidly renders the membrane permeable and thus reaches the embryo. By the same

¹ J. Loeb: *Science*, xxxvi, p. 637, 1912; *Biochem. Ztschr.*, xlvii, p. 127, 1912.

method it was ascertained that after a brief treatment of the egg with an injurious solution it sank very rapidly when put into a mixture of 100 cc. 3 M NaCl + 1 cc. 10/8 M CaCl_2 .

This method could not be applied to the fish itself and it seemed advisable to ascertain for this object by a direct test whether or not a pure NaCl solution of a sufficient concentration increases the permeability of the fish rapidly, while the addition of a trace of CaCl_2 counteracts this effect. This question could be decided by measuring the concentration of the body juice of the fish in isosmotic concentrations of balanced (*e.g.*, sea water or NaCl + CaCl_2) and non-balanced solutions (*e.g.*, NaCl).

Fundulus is too small to permit extensive work with the determination of the alteration of the freezing point of the blood. It was necessary to determine the alterations of the freezing point of the body juice of the fish, obtained with the aid of a Buchner press. The general method consisted in this: The fish were kept in a solution of a certain concentration and constitution for some time, and then, after having been washed several times for about five minutes in a porcelain filter with fresh water (to get rid of the solutions adhering to the surface of the fish and its gills), they were decapitated, cut into smaller pieces, and submitted to the Buchner press. The freezing point of the liquid obtained was determined.

II.

The fish were kept in artificially prepared sea water consisting of 100 molecules of NaCl, 2.2 molecules of KCl, 1.5 molecules of CaCl_2 , 3.8 molecules of MgSO_4 , and 7.8 molecules of MgCl_2 . Since the sea water had to be prepared in large quantities no chemically pure salts were used. The freezing point depressions of this sea water for different concentrations were as follows:

	°C.
M/2 sea water	$\Delta = 2.071$
M/4 sea water	$\Delta = 1.038$
M/8 sea water	$\Delta = 0.540$
M/16 sea water	$\Delta = 0.285$

The fish were kept in M/2 sea water unless the contrary is stated. The pure NaCl solutions used had the following freezing point depressions:

	°C.
M/2 NaCl	$\Delta = 1.780$
M/4 NaCl	$\Delta = 0.895$

We took it for granted that the freezing point depression of the body juice of *Fundulus* kept in M/2 sea water would be a constant, but were surprised to find that this is not the case. It was generally noticed that the freezing point of the body juice of the fish became lower the longer they were kept in the laboratory. Thus in one case the body juice of the fish, immediately after they were brought into the laboratory and before they were put into the tanks, had a depression of $\Delta = 0.945^\circ$. The fish were then distributed into three solutions of artificial sea water of different concentrations, M/2, M/8, and M/16. After three intervals of 1, 8, and 21 days the freezing point of their body juice in each of these solutions was determined.

TABLE I.

	DEPRESSION OF FREEZING POINT OF BODY JUICE OF FISH AFTER		
	1 day	8 days	21 days
	°C.	°C.	°C.
In M/2 sea water	1.050	0.925	0.830
In M/8 sea water	0.900	0.935	0.825
In M/16 sea water	0.900	0.840	

In M/2 sea water the concentration of the body juice of the fish was diminished more than 25 per cent in three weeks. In M/8 sea water at first a slight rise but then also a diminution took place. In a second experiment of this kind the body juice of the fish when brought into the laboratory, before they were put into the M/2 sea water, had a freezing point depression of $\Delta = 0.875^\circ$. They were then put into M/2 and M/8 sea water, respectively.

TABLE II.

	DEPRESSION OF FREEZING POINT OF BODY JUICE OF FISH AFTER	
	3 days	16 days
	°C.	°C.
In M/2 sea water	0.925	0.800
In M/8 sea water	0.895	0.785

Again we notice that the freezing point depression becomes less the longer the fish remain in the laboratory. The difference in $M/2$ and $M/8$ sea water was small, as was to be expected since *Fundulus* lives equally well in $M/2$ and $M/8$ sea water. The body juices of the fish become less concentrated the longer the fish lives in the aquarium, and this diminution therefore is in wide limits independent of the concentration of the outside solution. We thought at first that we were the victims of definite errors due to differences in the way of washing the fish, but control experiments eliminated this explanation. It finally occurred to us that the lack of food might be the cause of this change, since the fish were not fed in the laboratory. A test seemed to confirm this suspicion. A set of fish brought into the laboratory on November 23 were kept under the same conditions and without food, each in $M/2$ sea water. After three weeks the freezing point depression of their body juice was found to be 0.795° . They were then divided into two lots; one was fed regularly with fish food, the other remained unfed. The result is contained in Table III.

TABLE III.

FREEZING POINT DEPRESSION OF FISH IN $M/2$ SEA WATER AFTER	FED	UNFED
	$^{\circ}C.$	$^{\circ}C.$
2 days.....	0.820	0.795
8 days.....	0.890	0.740

After eight days of feeding the freezing point depression had increased considerably, while in those not fed the freezing point had diminished further.

It is not possible to express any suggestion as to the source of the effect which starving has on the freezing point depression, though it seems more promising to look for the cause of these variations in the organic rather than the inorganic soluble constituents of the fish. As a result of our observations we may state that the osmotic pressure of the body juice of *Fundulus* in $M/2$ or $M/8$ sea water is not a constant, but diminishes apparently with continued starvation. It varies roughly between $\Delta = 1.00^{\circ}$ and $\Delta = 0.78 - 0.74^{\circ}$ or a little less. Roughly speaking, it is a little below that of $M/4$ sea water and may go 25 per cent below this limit.

The results show that in experiments of this kind the freezing point of the body liquid in normal sea water has to be determined as a control in each case, especially if the fish are not fed as in our experiments.

III.

The Influence of Varying Concentrations of the Sea Water upon the Osmotic Pressure of the Body Juices of Fundulus.

Loeb has shown that this fish can live equally well in solutions from 10/8 M to M/128 sea water.² It is necessary to bring the fish not too suddenly from normal into the 10/8 M sea water. It was of interest to find out the possible changes which occur in the concentration of the body juices of these fish in sea water of different concentrations. Fish brought in at the same time were distributed into various dilutions of sea water. Table IV gives the result in dilute solutions.

Fundulus were kept for eight days in sea water of the following concentrations: M/2, M/4, M/16, M/32, M/64, and M/128. The osmotic pressure of the body juice was determined after one day, and after eight days. (The fish had been in the laboratory for eighteen days previous to the beginning of the experiment.)

TABLE IV.

	Δ AFTER 1 DAY	Δ AFTER 8 DAYS
	°C.	°C.
M/2 sea water	0.850	0.790
M/4 sea water	0.810	0.820
M/8 sea water	0.780	0.760
M/16 sea water	0.755	0.735
M/32 sea water	0.770	0.770
M/64 sea water	0.765	0.730
M/128 sea water	0.715	0.750

The influence of the dilution of the sea water is more marked after one day than after eight days. If we make allowance for irregularities due to unknown internal causes we notice that there is after eight days only a slight if any difference between the body liquids of the fish kept in sea water varying between M/2 and M/128, and this

² Loeb: *Biochem. Ztschr.*, liii, p. 391, 1913.

agrees with the fact that in all these solutions the fish can live indefinitely with the possible exception of the weakest solution.

The statement occasionally made that the blood of euryhaline³ fish has a lower osmotic pressure when they are in dilute sea water is not correct for the body liquid of *Fundulus*, when this teleost has been kept for some time in the medium; while a slight influence of the concentration may show itself earlier.

With the same lot of fish some experiments had previously been made with sea water of a higher concentration; namely, 6/8 M and 10/8 M, after one day and later. All the fish had first been in M/2 sea water and the concentration of the sea water was gradually raised (each day by almost but not quite M/8) until it had the desired concentration.

TABLE V.

	Δ AFTER 1 DAY	Δ AFTER 6-8 DAYS	Δ AFTER 9 DAYS
	°C.	°C.	°C.
M/2 sea water	0.925	0.850	
6/8 M sea water	1.025	0.880	
8/8 M sea water	1.190	0.945	
M/2 sea water	0.850	0.800	0.825
10/8 M sea water	1.190	1.195	1.040

In another experiment after one day Δ was in 6/8 M sea water 0.915; after five days 0.855.

For the sake of completeness we wish to add a series of measurements made two years ago.

TABLE VI.

Lowering of the Freezing Point of the Body Juice of Fundulus.

	Δ °C.
In M/80 NaCl + KCl + CaCl ₂ after 4 days	1.01
In M/80 NaCl + KCl + CaCl ₂ after 1 day	0.97
In M/80 NaCl + KCl + CaCl ₂ after 1 day	0.99
In M/8 NaCl + KCl + CaCl ₂ after 7 days	0.95
In M/2 NaCl + KCl + CaCl ₂ after 7 days	0.98
In M/2 sea water after a number of days	1.00
In M/2 sea water after a number of days	1.00
In M/2 sea water after a number of days	0.96
In M/2 sea water after a number of days	1.01
In 10/8 M NaCl + KCl + CaCl ₂ adapted gradually	1.12

³ This term denotes the fact that the fish can live in concentrations varying within wide limits.

The results agree with those given before, except that Δ was in general slightly higher, possibly because the fish had been fed regularly, though we have failed to keep a record on this point.

The result is very characteristic. During the first day the body juices of the fish change in concentration in the same sense with the change in the concentration of the medium. After some time this difference disappears or is greatly diminished. This diminution is less noticeable in the 10/8 M sea water than in the M/1 or 6/8 M sea water. This may possibly be explained by the fact that the fish were kept at a comparatively high temperature and hence were not quite normal in 10/8 M sea water. This point will be further investigated. It is, therefore, possible that the change in the concentration of the sea water increases transitorily the permeability of the skin, but that this transitory increase in permeability is rapidly repaired and leads to no injury of the fish.

IV.

We now come to the main problem of this paper; namely, whether the permeability is increased more if we put the fish into a non-balanced solution than if we put it into a balanced solution of the same concentration. For the sake of briefness we consider a solution of one sodium salt, *e.g.*, NaCl or NaBr or Na₂SO₄, as a non-balanced solution, while the addition of a trace of CaCl₂ makes the solution more balanced. NaCl + KCl + CaCl₂ in the proportion in which these salts exist in the sea water is more balanced than NaCl + CaCl₂ without KCl, and sea water is a completely balanced solution. It should also be realized that as long as a pure NaCl solution has a very low concentration it is not very toxic, *i.e.*, the fish can live very long if not indefinitely in such a solution, and hence it is not possible to show that the addition of CaCl₂ improves the solution, though this may be the case. If, however, the concentration of NaCl increases, it becomes more toxic, and in this case the addition of CaCl₂ prolongs the life of the fish considerably. It should also be remembered that the toxic limit of the solution of different Na salts varies with the anion; thus Na₂SO₄ is more toxic than NaCl, and NaNO₃ more toxic than either; *i.e.*, it requires a

lower concentration of Na_2SO_4 , and a still lower concentration of NaNO_3 , to kill the fish in a definite time.

The theory formerly expressed by Loeb consisted in this, that NaCl (or Na_2SO_4 or NaNO_3) should increase the permeability of the skin of the fish more rapidly when alone in solution than when CaCl_2 is added. When, therefore, the toxic solution is hypertonic (compared with the concentration of the liquids of the body) the concentration of the body juices of the fish should be increased more rapidly in a pure solution of NaCl than in a mixture of $\text{NaCl} + \text{CaCl}_2$ of the same osmotic pressure; and if the toxic concentration has a lower concentration than that of the liquids of the body the decrease in the concentration of the body juices of the fish should be more rapid in the pure solution of the toxic salt than if an antagonistic salt is added.

Moreover, it should be expected that the toxic solution gradually increases the permeability, and that the higher the concentration of a given toxic solution, the more rapidly should the permeability increase. The change in the permeability precedes the change in the concentration of the body liquid.

In a pure $6/8$ M NaCl solution the fish will die in a few hours; in an $8/8$ M solution they will die in less than one-half hour. In a $M/2$ NaCl solution they may live a day; sometimes a few may live two days or a little longer.

	°C.
Δ of fish 0.5 hour in $M/1$ NaCl (all dead)	1.490
Δ of fish 1.5 hours in $6/8$ M NaCl (1 normal, 4 sick, 1 dead)	1.410
Control: Δ of fish in $M/2$ sea water	0.950

Another experiment of the same kind gave the following result :

	°C.
Δ of fish 0.5 hour in $8/8$ M NaCl (4 normal, 4 lying on side)	1.255
Δ of fish 1.5 hours in $6/8$ M NaCl (4 normal, 5 lying on side)	1.270
Control: Δ of fish in $M/2$ sea water	0.820

The rise of concentration of the body juice of the fish in a $6/8$ M and $8/8$ M NaCl solution is therefore enormous.

The experiment with a $6/8$ M NaCl solution was repeated twice with the same result.

	°C.
Δ of fish 2 hours in $6/8$ M NaCl (3 normal, 1 sick)	1.160
Δ of fish 3 hours in $6/8$ M NaCl (3 normal, 1 sick)	1.135

In one experiment in $M/2$ NaCl the result was as follows:

	°C.
Δ of fish 24 hours in $M/2$ NaCl	1.095
Δ of fish 42 hours in $M/2$ NaCl	1.160
Δ of fish 24 hours in $M/2$ sea water	1.020

The concentration of the body juice of the fish therefore is higher in $M/2$ NaCl than in $M/2$ sea water, and it rises the more the longer the fish remains in the $M/2$ NaCl solution, indicating that the change of the concentration of the liquid of the body accompanies or follows the alteration of the surface of the body. *In all these experiments it was obvious that as soon as the fish began to lie on their sides the osmotic pressure of their body juices had increased.* The toxic effect of the solutions and the increase in permeability were closely associated.

The question may be asked whether in these experiments the increase in osmotic pressure noticed in such fish when kept in hypertonic NaCl solution was not the consequence of this condition of sickness. The same type of sickness can be produced quickly if we cause the fish to suffocate by replacing the air in the solution by hydrogen. Control experiments showed that fish rendered "sick" in a pure NaCl solution by driving out the air from the solution did not show any greater alteration of the freezing point of their body juices than fish in the same NaCl solution which had remained in contact with air and which were still normal. Hence it is obvious that the fact that the fish began to lie on their sides in the pure NaCl solutions was the consequence but not the cause of the increased permeability of their skin.

We now wish to compare with this the behavior of the fish in physiologically balanced solutions.

Experiment 1.

	°C.
Δ of fish 1.5 hours in $6/8$ M NaCl (2 normal, 2 lying on side)	1.350
Δ of fish 1.5 hours in 100 cc. $6/8$ M NaCl + 1 cc. $6/8$ M $CaCl_2$ (all normal) ..	1.010
Δ of fish 1.5 hours in $6/8$ M sea water	1.070
Δ of fish 1.5 hours in $M/2$ sea water	0.990

Experiment 2.

	°C.
Δ of fish 1.5 hours in $6/8$ M NaCl (all but 1 normal)	1.110
Δ of fish 1.5 hours in $6/8$ M sea water (all normal)	0.890
Δ of fish 1.5 hours in $M/2$ sea water	0.835

Experiment 3.

	°C.
Δ of fish 2 hours in 6/8 M NaCl (5 normal, 3 sick)	1.320
Δ of fish 2 hours in 6/8 M sea water (all normal)	0.990

It is obvious that in hypertonic *physiologically balanced solutions the concentration of the body juice of Fundulus rises considerably less than in the non-balanced solutions of the same concentration.*

We will now take up experiments with weaker solutions in which the toxic effects appear much later.

Experiment 4.

	°C.
Δ of fish 4 hours in 5/8 M NaCl (all normal)	1.105
Δ of fish 4 hours in 100 cc. 5/8 M NaCl + 1 cc. 5/8 M CaCl ₂ (all normal) ..	0.975

Experiment 5.

	°C.
Δ of fish 20 hours in M/2 NaCl	1.050
Δ of fish 22 hours in 100 cc. M/2 NaCl + 1 cc. M/2 CaCl ₂	0.950

In these and weaker solutions the difference in the action of the non-balanced and balanced solution became marked when the injurious effects of the non-balanced solution began to show themselves. It was therefore necessary to compare the effect of pure NaCl solutions of lower concentrations than M/2 for different periods of time.

Experiment 6.

	°C.
Δ of fish 17 hours in 3/8 M NaCl (all normal)	0.860
Δ of fish 18.5 hours in 100 cc. 3/8 M NaCl + 1 cc. 3/8 M CaCl ₂ (all normal) ..	0.850
Δ of fish 3 days in 3/8 M NaCl (1 died, rest normal; only the normal fish used for determination)	1.085
Δ of fish 3 days in 100 cc. 3/8 M NaCl + 1 cc. 3/8 M CaCl ₂ (all normal) ...	0.915

After three days, when the toxic effect of the pure NaCl solution began to show itself, the influence on the concentration of the body juice was also marked.

Experiment 7.

	°C.
Δ of fish 20 hours in M/4 NaCl (all normal)	0.880
Δ of fish 22 hours in 100 cc. M/4 NaCl + 1 cc. M/4 CaCl ₂ (all normal)	0.875
Δ of fish in M/2 sea water	0.855
Δ of fish 4 days in M/4 NaCl (all normal)	{ a..... 1.000 b..... 0.970
Δ of fish 4 days in 100 cc. M/4 NaCl + 1 cc. M/4 CaCl ₂ (all normal)	0.860

In a $M/4$ solution of NaCl only after about four days will the injurious effects appear, and at this time we also notice that in the pure $M/4$ solution of NaCl the osmotic pressure of the body juice of the fish increases. Some of the NaCl apparently enters the fish.

In pure solutions of $M/8$ NaCl or below this concentration *Fundulus* lives for weeks or months (if not indefinitely), which means in the sense of Loeb's theory that in such a solution the permeability of the surface of the fish is not perceptibly altered for such a period of time; and hence we should expect that in these cases the addition of $CaCl_2$ to an NaCl solution does not alter the permeability. This was found to be the case.

Experiment 8.

	°C.
Δ of fish 20 hours in $M/8$ NaCl (all normal)	0.815
Δ of fish 20 hours in 100 cc. $M/8$ NaCl + 1 cc. $M/8$ $CaCl_2$ (all normal)	0.795
Δ of fish 4 days in $M/8$ NaCl (all normal)	0.840
Δ of fish 4 days in 100 cc. $M/8$ NaCl + 1 cc. $M/8$ $CaCl_2$ (all normal)	0.775

V.

Experiments with Na_2SO_4 and NaBr.

The fish do not live long in a $M/4$ solution of Na_2SO_4 , and as a rule they soon die even in a $M/8$ solution of Na_2SO_4 ; $M/50$ or $M/20$ solutions are tolerated for a longer time. The following table gives the results of some of the experiments.

TABLE VII.

Δ OF BODY JUICE OF FUNDULUS AFTER		°C.
5 days in $M/50$ Na_2SO_4		0.815
6 days in $M/50$ sea water		0.815
48 hours in $M/20$ Na_2SO_4		0.765
50 hours in $M/20$ sea water		0.855
20 hours in $M/10$ Na_2SO_4		0.775
22 hours in $M/10$ sea water		0.875
2.5 hours in $M/4$ Na_2SO_4 (fish began to turn on side)		0.930
2.5 hours in $M/4$ sea water		0.820
1.5 hours in $M/2$ Na_2SO_4		1.125
1.5 hours in $M/2$ sea water		0.890

These experiments show a definite result. Whenever the osmotic pressure of the pure Na_2SO_4 solution is higher than the normal concentration of the body juice of the fish ($\text{M}/2$ and $\text{M}/4$ Na_2SO_4) it raises the osmotic pressure of the liquids of the fish more than is done by the corresponding concentration of sea water; whenever the concentration of the Na_2SO_4 solution is lower than the concentration of the body juices of the fish ($\text{M}/10$ Na_2SO_4 and less) the reverse is the case, although the difference is small. When finally the concentration of Na_2SO_4 becomes so low that it ceases to be toxic ($\text{M}/50$ Na_2SO_4) it behaves osmotically like sea water of the same concentration. This shows that a pure Na_2SO_4 solution of sufficiently high concentration makes the fish more permeable.

In a former paper Loeb has shown that the addition of CaCl_2 or $\text{CaCl}_2 + \text{KCl}$ renders a pure Na_2SO_4 solution less harmful. Accordingly we should expect that in the latter case the permeability is altered less than in the former case.

TABLE VIII.

Δ OF BODY JUICE OF FUNDULUS AFTER		$^{\circ}\text{C}.$
Hypertonic solutions	3 hours in $\text{M}/4$ Na_2SO_4 (fish normal)	0.910
	3 hours in 100 cc. $\text{M}/4$ $\text{Na}_2\text{SO}_4 + 1.5$ cc. $\text{M}/2$ $\text{CaCl}_2 + 2.2$ cc. $\text{M}/2$ KCl (normal)	0.880
	16.5 hours in $\text{M}/4$ Na_2SO_4 (fish lying on side but alive)	1.030
	18 hours in 100 cc. $\text{M}/4$ $\text{Na}_2\text{SO}_4 + 1$ cc. $\text{M}/2$ CaCl_2 (majority normal)	0.970

TABLE VIII.—*Concluded.*

Δ OF BODY JUICE OF FUNDULUS AFTER		$^{\circ}\text{C}.$
Hypotonic solutions	20 hours in $\text{M}/8$ Na_2SO_4 (mostly normal)	0.750
	20 hours in 100 cc. $\text{M}/8$ $\text{Na}_2\text{SO}_4 + 1$ cc. $\text{M}/2$ CaCl_2 (all normal)	0.810
	1 day in $2/25$ M Na_2SO_4 (all normal)	0.720
	1 day in 100 cc. $2/25$ M $\text{Na}_2\text{SO}_4 + 0.75$ cc. $\text{M}/2$ CaCl_2 (all normal) ..	0.860
	3 days in $2/25$ M Na_2SO_4 (all normal)	0.800
	3 days in 100 cc. $2/25$ M $\text{Na}_2\text{SO}_4 + 0.75$ cc. $\text{M}/2$ CaCl_2 (all normal) ..	0.840
	1 day in $\text{M}/20$ Na_2SO_4	0.770
	1 day in 100 cc. $\text{M}/20$ $\text{Na}_2\text{SO}_4 + 0.75$ cc. $\text{M}/2$ CaCl_2	0.825
	4 days in $\text{M}/20$ Na_2SO_4	0.765
	4 days in 100 cc. $\text{M}/20$ $\text{Na}_2\text{SO}_4 + 0.75$ cc. $\text{M}/2$ CaCl_2	0.820

These experiments prove that the addition of CaCl_2 diminishes or inhibits the increase of permeability induced by weak solutions of Na_2SO_4 . The effect, however, is slight.

Very low concentrations of NaBr solutions cause the animals to lie on one side and ultimately cause their death, while the addition of CaCl_2 inhibits this effect. Since all these solutions are strongly hypotonic it was to be expected that if CaCl_2 is added to the solution the concentration of the body juices of the fish is higher than without the addition of CaCl_2 . This is the case.

TABLE IX.

Δ OF BODY JUICE OF FUNDULUS AFTER		$^{\circ}\text{C}$.
1 day in $\text{M}/50$ NaBr (all normal)		0.820
1 day in 100 cc. $\text{M}/50$ NaBr + 0.75 cc. $\text{M}/2$ CaCl_2 (all normal)		0.860
3 days in $\text{M}/50$ NaBr (all lying on side)		0.750
3 days in 100 cc. $\text{M}/50$ NaBr + 0.75 cc. $\text{M}/2$ CaCl_2 (all normal)		0.825
1 day in $\text{M}/25$ NaBr (all normal)		0.830
1 day in 100 cc. $\text{M}/25$ NaBr + 0.75 cc. $\text{M}/2$ CaCl_2 (all normal)		0.845
3 days in $\text{M}/25$ NaBr (all lying on side)		0.790
3 days in 100 cc. $\text{M}/25$ NaBr + 0.75 cc. $\text{M}/2$ CaCl_2 (all normal)		0.840

VI.

These experiments do not all give an equally positive answer to the question whether or not physiologically balanced solutions increase the permeability of the fish. The answer was most strikingly positive in the case of hypertonic NaCl solutions. The answer seemed positive but was certainly less clearly so in the case of Na_2SO_4 or NaBr solutions (and we may also add in the case of NaNO_3 , KCl , and of acid, though we are not giving the figures in this paper).

We can understand the fact if we bear in mind that although the non-balanced solutions alter the permeability, the death of the fish is not due to a change in the osmotic pressure of the body juices but to another effect of these substances, which requires only a slight alteration and slight increase of permeability of the epithelium of the gills, without altering the water contents of the fish perceptibly. The writers, in a former paper, examined the toxic effects of acid on fish

and came to the conclusion that very weak acid solutions kill the fish by making the gills unfit for respiration (*e.g.*, by causing an excessive mucus secretion or by otherwise agglutinating the gill leaves).⁴ This presupposes an alteration of the epithelium of the gills, but the fish die from suffocation long before any greater osmotic changes than those taking place in a neutral H_2O solution have had time to occur.

When the fish is put into a pure solution of NaNO_3 , or NaBr , or KCl , etc., traces of these salts probably diffuse to the nerves or muscles of the gill or into the internal ear and possibly along the auditory nerve to the medulla. We may expect in this way a toxic action of the salt long before a great change in the osmotic pressure of the body juices has taken place. According to our figures, a slight increase in the permeability of the epithelium of the gills precedes this effect of Na_2SO_4 , NaBr , etc., on the nerve elements which is prevented through the addition of CaCl_2 . It is not excluded, however, that the antagonistic action of CaCl_2 in this case is not entirely due to a lowering of the permeability. Only in the case of the hypertonic solutions of NaCl do we notice great osmotic changes in the fish before it is killed. This is due in our opinion to the fact that the direct toxic effects of this salt on the nerves and muscles take place only when its concentration in the gills or in the brain cavity is comparatively high.

It is, therefore, obvious that death in all these cases is not due to the increase in the concentration of the body juice but to the toxic salts which diffuse into the gills (or the internal ear and brain cavity?) and which kill the fish by stopping respiration through influencing the medulla or the nerves or muscles of the gills. But in all cases the diffusion of these salts to the nerves or muscles of the gill or the internal ear or the brain cavity presupposes an increase of the permeability of the gills, although this can not be proved in all cases.

Fundulus differs from most fish in that it is within wide margins independent of the osmotic pressure of the sea water; but it is rapidly killed when suddenly put from normal sea water into sea water of a concentration above $9/8$ M. Our experiments seem to indicate that

⁴ J. Loeb and H. Wasteneys: *Biochem. Ztschr.*, xxxix, p. 167, 1912.

such solutions also increase the permeability of the epithelium of the gills or the whole fish. In other words, a balanced solution ceases to be balanced when its concentration exceeds a certain limit. This is sufficiently intelligible on the basis of Loeb's former experiments which showed that CaCl_2 can antagonize a solution of any other salt only as long as this solution does not exceed a certain concentration. For *Fundulus* this limit is high when NaCl is the toxic salt. For other fish the limit is much lower, and they can stand sea water, which is a physiologically balanced solution, only in very low concentrations. This is the case with the majority of fresh water fish, which die rapidly when put into ordinary ($M/2$) sea water: Paul Bert found that such fish die from suffocation and that their death is caused by "an exosmotic effect upon the gills whose epithelium becomes pale and where the circulation stops. The salt of the sea water withdraws water from the epithelium and the proper tissue of the gills and the finest ramifications of the blood vessels are obliterated, either by direct action upon the surrounding tissue and their contractile fibers or reflexly through the vasomotor nerves."⁵ In addition thrombosis might occur. Backman has recently published some observations on the behavior of marine dogfish when put into hypotonic solutions. He found that if the sea water in which these fish live (with $\Delta = 1.880^\circ$) is diluted to $\Delta = 0.5^\circ$, after fifteen minutes the tension of O_2 in the blood is diminished from 19.1 to 3.7 per cent O_2 . He assumes that "the hypotonic medium probably leads to a rapid injury of the gills whereby the absorption of O_2 is inhibited considerably."⁶ For these fish sea water in a lower concentration probably alters the epithelium of the gills by causing cytolysis (?).

SUMMARY.

1. The experiments published in this paper show that the concentration of the body juice of *Fundulus* is influenced more strongly by non-balanced solutions than by balanced solutions of the same concentration. This supports the theory of Loeb that non-balanced solutions increase the permeability of the cells.

⁵ P. Bert: *Compt. rend. Acad. d. sc.*, lxxiii, pp. 382 and 464, 1871; xcvi, p. 133, 1883.

⁶ E. L. Backman: *Zentralbl. f. Physiol.*, xxviii, p. 495, 1914.

2. This influence of the non-balanced solutions on the concentration of the body juice of *Fundulus* is more obvious when the non-balanced solution is a pure (hypertonic) NaCl solution than when it is a solution of any other salt (Na_2SO_4 , NaBr, etc.).

3. This finds its explanation in the fact that the fish do not die in a non-balanced solution from the change in the osmotic pressure of their body juice but from the action of the toxic salt on the epithelium, nerves, and muscles of the gills, or by action on the inner ear and medulla, which interferes with and finally stops the respiration. In the case of a very toxic salt the fish is killed before a marked change in the osmotic pressure of the body juice has taken place; while in the case of the less toxic NaCl great osmotic changes may take place in the fish before the salt will kill it.

4. Balanced solutions, *e.g.*, sea water, when applied in excessive concentrations, also increase the osmotic pressure of the body juice of the fish; possibly by first increasing the permeability of the gills (and the skin) of the fish.

5. When *Fundulus* are kept in $M/2$ sea water for some length of time the osmotic pressure of their body juice diminishes constantly to a certain limit, apparently as a consequence of starvation, since feeding seems to inhibit this decline.

THE RELATIVE EFFICIENCY OF VARIOUS PARTS OF THE SPECTRUM FOR THE HELIOTROPIC REAC- TIONS OF ANIMALS AND PLANTS.

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I. INTRODUCTION.

While the older authors had treated the motile reactions of animals to light as an indication of their love for or antipathy to this kind of energy, one of us in 1888 pointed out that we are dealing in these cases with phenomena of orientation comparable to the orientation of plants to light.¹ In order to indicate the identity of the mechanism in both cases he proposed the same term for both, namely, heliotropism (or phototropism). Loeb stated in his first full pamphlet (1889)² that (if one source of light be given) the animals orient themselves so that their plane of symmetry falls into the direction of the rays of light, "whereby the symmetrical points of the surface of the body are struck by the light at the same angle." In 1897 the same writer expressed the idea that the action of light which caused the heliotropic reactions was chemical.³ Since it is reasonable to assume that symmetrical elements of the surface of the body are not only morphologically but also chemically alike, we must suppose that if the symmetrical elements of the surface of the animal are struck by the rays of light at the same angle, the velocity of the photochemical reactions in symmetrical elements of the surface (e.g., the eyes or skin) are the same, since the intensity of the illumination of a surface element varies with the cosine of the angle of incidence.

¹ Loeb, J. Sitzungsab. d. Würzburger physik.-med. Gesellsch., 1888.

² Der Heliotropismus der Tiere und seine Übereinstimmung mit dem Heliotropismus der Pflanzen. Würzburg, 1889.

³ Loeb, J. Zur Theorie der physiologischen Licht- und Schwerkraftwirkungen. Pflüger's Archiv, Bd. 66, p. 439, 1897.

The influence of the light upon the tension and action of symmetrical muscles must in such a case be identical. The light, if it remains constant, will therefore not cause the animal to alter the direction of its motions. If, however, the light strikes symmetrical elements at a different angle the velocity of photochemical reaction is not the same in the two symmetrical elements and the symmetrical muscles on both sides of the animal will receive unequal impulses (by reflex) from this source. This will lead to an automatic turning of the animal until its plane of symmetry again falls into the direction of the rays of light.

If these premises were true, it followed that the heliotropic reactions of animals should obey the law of Bunsen and Roscoe which says that (within certain limits) the photochemical effect of light is equal to the product of the intensity into the duration of illumination; and one of us predicted that this law would probably be found to hold for animal heliotropism.⁴ This prediction proved true for the heliotropic curvatures of *Eudendrium*, as the experiments of Loeb and Ewald⁵ showed. Ewald could also show that Talbot's law holds for the orientation of the eye of *Daphnia* by light,⁶ and Talbot's law is an expression of the fact that the physiological effect of light is equal to the product of intensity and duration of illumination.

The same law holds for the heliotropic reactions of plants, as Blaauw and Fröschl had shown.⁷ It also holds for the human eye.⁸ In all these cases it should be remembered that the law of Bunsen and Roscoe is a threshold law, inasmuch as it holds only within certain limits.

With the reduction of both groups of heliotropic reactions, those of animals as well as of plants, to the same law, namely, that of Bunsen and Roscoe, it is idle to consider further the idea that animals are led to the light because they are "fond" of it, or that they are turned away from it because they hate it; or that the reactions are the result of "trial and error."

⁴ Loeb, J. *The mechanistic conception of life*. Chicago, 1912.

⁵ *Zentralbl. f. Physiol.*, Bd. 27, p. 1165, 1914.

⁶ *Science*, N. S., vol. 38, p. 236, 1913.

⁷ Blaauw, *Rec. des Travaux Botaniques Néerlandais*, Bd. 5, p. 209, 1909; Fröschl, *Sitzungsb. d. Akad. in Wien*, 1908.

⁸ Charpentier, *Arch. d'Ophthalmol.*, tom. 10, 1890.

We may, therefore, conclude that the heliotropic reactions of animals and plants are due to photochemical reactions and that the turning of the animal to (or from) the source of light is brought about automatically if the velocity of photochemical reaction is no longer the same in symmetrical areas of the photosensitive surface. This automatic turning results when the mass of photochemical reaction products on symmetrical points of the surface of the animal (eyes or skin) exceeds a certain value; and the variations of this value determine the relative sensitiveness of different heliotropic animals. Since this has been stated more fully in former publications of Loeb we may refer the reader to these publications.⁹

If the basis of heliotropic reactions is a photochemical process, it follows that heliotropic animals must possess a photosensitive substance, and the question arises: Is this substance identical in all heliotropic organisms or do the photochemical substances differ in different heliotropic organisms? Especially does this question become of interest in respect to the question whether there is a specific difference between these substances in animals and plants.

The method to decide this question consists in comparing the relative heliotropic efficiency of different wave lengths in different organisms. If we find that the optimal heliotropic effects occur for one form of organisms in one kind of wave lengths, for another in a widely different wave length of the same spectrum, we may conclude that the photochemical substances in the two cases are different, if deduction be made for the possible screen effect of secondary substances contained in the sensitive organ.

The older experiments of the botanists were mostly made with colored screens, which yielded the result that behind red screens only weak or no heliotropic reactions of plants occur, while behind blue screens they occur as well as in mixed daylight. Loeb was able to show in his earlier experiments that the same holds good for the heliotropic reactions of animals. But these experiments are not adequate to decide the question of perfect identity of the photochemical substances in all cases. For this purpose experiments with spectral colors are required. The most reliable experiments made

⁹ The mechanistic conception of life. Chicago, 1912; article on Tropisms in Winterstein's *Handbuch der vergleichenden Physiologie*, Bd. 4, p. 451, 1912.

on plants are apparently those of Blaauw on the spore bearers of *Phycomyces* and the seedlings of *Avena*.

Blaauw proceeded in the following way: He exposed a row of seedlings of *Avena* to a carbon arc spectrum for a certain time. The seedlings were then placed in the dark and after the proper time it was ascertained which part of the spectrum had induced heliotropic curvatures. By varying the duration of time of exposure to the spectrum it was found that with a minimal time of exposure only certain blue rays, namely, those of a wave length of $478\ \mu\mu$, caused heliotropic bending, while with longer exposure longer waves also became efficient. In this way the minimum duration of exposure for various parts of the spectrum was ascertained. Table I gives his result.

TABLE I:

DURATION OF ILLUMINATION, IN SECONDS	LOCATION OF THRESHOLD IN THE SPECTRUM, IN MICRA
6300	534
1200	510
120	499
15	491
5	487
4	478
3	...
4	466
6	448

The red and yellow parts of the spectrum were ineffective for the intensity and time limits used and the optimum of efficiency was in the blue, in the region between 466 and $478\ \mu\mu$.

A shorter series of experiments was made on the fruit bearers of *Phycomyces*, with the following results:

44 to 47 per cent of the *Phycomyces* showed heliotropic curvatures

- after 192 seconds of illumination at $615\ \mu\mu$
- after 192 seconds of illumination at $550\ \mu\mu$
- after 16 seconds of illumination at $495\ \mu\mu$
- after 32 seconds of illumination at $450\ \mu\mu$
- after 64 seconds of illumination at $420\ \mu\mu$

The number of experiments was limited but they indicate an optimum between 495 and $450\ \mu\mu$, in this respect agreeing with the results on *Avena*.

We were anxious to know whether for the heliotropic reactions of sessile animals the optimum is situated in the same region of the spectrum. The number of sessile animals which are sensitive to light is rather limited and we had to make use of the hydroid *Eudendrium*, which also served in the experiments of Loeb and Ewald.

II. THE HELIOTROPIC REACTIONS OF EUDENDRIUM.

The newly formed polyps are positively heliotropic to light and they react by bending towards the light. The bending occurs in the region near the stem; the method of procedure was as follows:

Immediately after the colonies were brought into the laboratory good stems with from 4 to 8 or more polyps were selected. The polyps were cut off and the stems put into glass troughs filled with sea-water, where they were held in position by being fixed in little holes of a layer of paraffin, on the bottom of the trough. The troughs had plain parallel walls. The stems were exposed during the first day to ordinary light—since light is necessary for the regeneration of polyps¹⁰—and were then put into the dark-room. In the dark-room the polyps developed during the next day. These newly formed polyps are very sensitive to light and were used for the experiment. The trough was then exposed to a carbon arc spectrum, the visible portion of which was about 20 cm. wide. The spectrum was in a dark-room and all precautions were taken to guard against any reflected or other light from reaching the polyps. The stems were in a row and each one was exposed to a different part of the spectrum. The position of each individual polyp was marked in a diagram at the beginning of the experiment and the polyps were exposed to the spectrum for times varying from five minutes to five hours. Then the polyps were put into the dark again and after the proper time (two hours or more) the number of the polyps which had bent to the light in various parts of the spectrum was ascertained. As the reader will notice, the bending of the polyps took place after they had been put back into the dark. This corresponds with the method followed by Blaauw in his experiments on plants and of Loeb and Ewald in their experiments on the applicability of the law of Bunsen and Roscoe to heliotropic reactions.

¹⁰ Loeb, J. Einfluss des Lichtes auf die Organbildung bei Tieren. *Pflüger's Archiv*, Bd. 63, p. 273, 1896.

We have also made experiments in which the stems were exposed long enough to the spectrum to enable them to form their polyps while still exposed to the light. The determination of the wave length to which each stem was exposed was rendered possible through the use of the absorption bands of a solution of didymium nitrate. With the aid of these bands, we could define accurately the position of each stem and polyp in the spectrum. We are indebted to Dr. E. Butterfield for the exact location of these bands in the spectrum.

In noting the result the reader must keep the following facts in mind: When a short exposure to light influences the orientation of the polyps of a stem, this will show itself in the fact that the majority of the polyps of that stem will bend straight to the light. If there is no effect of light, the polyps will grow in any direction but it may happen according to the laws of probability that one or the other may bend to the light. In order to be sure that the light influences the direction in which the polyps bend we must require that so great a percentage bend toward the light that chance may be excluded, before we draw the conclusion that we are dealing with a heliotropic effect. We considered it a positive result when 50 per cent or more of the polyps bent to the light. That they should all bend to the light cannot well be expected, especially in cases of short duration of exposure. The new polyps are extremely delicate and they are not all healthy or strong. Moreover, certain polyps will be partly screened from the light by the stems. Blaauw, as well as Loeb and Ewald, had to use the bending of 50 per cent of the specimens as a criterion of a positive result. The fact that each stem has only a limited number of polyps creates an additional difficulty, in this way: that if by chance one polyp is directed to the light and there are only five polyps on the stem it may appear as if 20 per cent of the polyps had reacted positively, while in reality the stem was not influenced by light at all. To avoid misinterpretations of this kind from influencing the interpretation of results we always give the number of polyps in a stem in the following tables.

In indicating the wave length it should always be remembered that the region given is usually the center of a small zone which contained the stem; since the latter is not straight and since the

polyps are irregular in position it is not possible to indicate the position by one line in the spectrum.

We will now enumerate some experiments. In the first vertical column is given the wave length, in Ångström units, to which the stems were exposed (indicating in the next column the color of the region). In the third column we give the fraction of the number

TABLE 2.

Experiment 1: Exposure of Eudendrium Polyps for Five Minutes to the Spectrum.

WAVE LENGTH IN ÅNGSTRÖM UNITS	COLOR OF THE SPECTRAL REGION	FRACTION OF POLYPS BENT TO THE LIGHT	PERCENTAGE OF POLYPS BENT TO THE LIGHT
About 6500	orange-red	1/29	(4)
About 6000	yellow	0/4	0
About 5700	yellow	0/13	0
About 5300-5345	yellowish-green	5/15	33
About 5100	green	3/12	25
About 4900	blue	11/32	35
About 4735	blue	30/49	62
About 4690	blue	4/21	19
4600	blue	5/22	23
4400	indigo	5/52	10

of polyps bending to the source of light over the total number of polyps. In the fourth column we give the percentage of the polyps bending to the light. Each experiment was made with different material on different days, unless the contrary is stated (table 2).

TABLE 3.

Experiment 2: Exposure, Five Minutes.

WAVE LENGTH IN ÅNGSTRÖM UNITS	COLOR OF THE SPECTRAL REGION	FRACTION OF POLYPS BENT TO THE LIGHT	PERCENTAGE OF POLYPS BENT TO THE LIGHT
About 5760	yellow	0/2	0
5711	green	1/2	(50)?
5100	bluish-green	4/6	66
4800	blue	9/22	41
4735	blue	3/3	100
4700	blue	3/11	27
4676	blue	8/10	80
4500	indigo	3/3	100
4400	indigo	2/2	100
	ultraviolet	0/11	0

From this experiment we may deduce, first, that for this duration of exposure (five minutes) the rays to 5700 Å.u. (i.e., the orange

and yellow rays) are absolutely ineffective; that the rays for 5300 to 4900 (i.e., yellowish-green, green, and greenish-blue) are only slightly effective; while the rays of the wave length of 4735 Å.u., (i. e., blue) constitute the *optimal* portion; and that in the indigo the efficiency diminishes again. This result is almost identical with the one obtained by Blaauw with the seedlings of oats (table 3).

TABLE 4.

Experiment 3: Duration of Exposure, Five Minutes.

WAVE LENGTH IN ÅNGSTRÖM UNITS	COLOR OF THE SPECTRAL REGION	FRACTION OF POLYPS BENT TO THE LIGHT	PERCENTAGE OF POLYPS BENT TO THE LIGHT
6200	orange	0/3	0
5600	yellow	0/5	0
5300	yellowish-green	1/10	(10)
5000	bluish-green	1/10	(10)
4850	blue	0/6	0
4735	blue	8/13	62
4700	blue	1/7	14
4450	indigo	0/3	0
4432	indigo	0/2	0
4000	violet	0/6	0
3850	violet	1/2	?
3600	ultraviolet	0/1	0

This result was less striking than the previous one through the combination of two circumstances: (1) The material was more sensitive than that used in the previous experiment, and (2) the number of polyyps was so small that the error was greater and the results not so uniform. Positive results were obtained in the region between Å.u. 4400 and 4735, that is, in the blue and indigo and also possibly in the bluish-green; yellow was ineffective, as before (table 4).

TABLE 5.

Experiment 4: Exposure to Light, Four Minutes.

WAVE LENGTH IN ÅNGSTRÖM UNITS	COLOR OF THE SPECTRAL REGION	FRACTION OF POLYPS BENT TO THE LIGHT	PERCENTAGE OF POLYPS BENT TO THE LIGHT
About 5600	yellowish-green	0/5	0
5400	yellowish-green	6/23	23
5000	bluish-green	4/22	18
4800	blue	4/13	31
4735	blue	18/30	60
4700	blue	2/12	17
4670	blue	9/21	43

The most effective region of the spectrum in this experiment is again the region about 4735 in the blue, the same which has proved the most effective in the two previous experiments. In all the experiments red, orange and yellow, and extreme indigo and violet, were ineffective.

TABLE 6.
Experiment 5: Exposure to Light, Three Minutes.

WAVE LENGTH IN ÅNGSTRÖM UNITS	COLOR OF THE SPECTRAL REGION	FRACTION OF POLYPS BENT TO THE LIGHT	PERCENTAGE OF POLYPS BENT TO THE LIGHT
5760	yellow	3/11	27
5600	yellow	1/13	8
5200	green	2/16	13
4900	blue	2/4	50
4800	blue	4/10	40
4735	blue	3/3	100
4700	blue	7/20	35
4676	blue	0/1	0
4500	blue	0/1	0
4432	indigo	3/12	25
4100	violet	0/2	0
4000	violet	1/7	14
3800	violet	0/2	0

The next two experiments (4 and 5, tables 5 and 6) were made with shorter exposure of the polyyps to the spectrum, namely, four and three minutes.

The exposure of three minutes is the minimum from which a result can be obtained, and the results of table 6 are difficult to account for. The time of exposure is so short that slight differences in the sensitiveness of various stems make themselves felt. Both experiments agree in their result with the previous ones, namely, that the region around 4735 Å.u. (in the blue) is the most efficient.

TABLE 7.
Experiment 6: Exposure to Light, Fifteen Minutes.

WAVE LENGTH IN ÅNGSTRÖM UNITS	COLOR OF THE SPECTRAL REGION	FRACTION OF POLYPS BENT TO THE LIGHT	PERCENTAGE OF POLYPS BENT TO THE LIGHT
3700-4100	extreme violet	14/30	45
4100-4900	violet-indigo and blue	72/95	76
4900-5400	green and bluish-green	14/37	38
5400-6700	orange-yellow to yellowish-green	0/?	0

It was to be expected that in a longer exposure polyyps would bend to the light in both indigo-blue and in green but not in yellow and

red. Table 7 gives the result of an experiment with an exposure of fifteen minutes.

Unfortunately, no record of the total number of polyps formed in the yellow and red was preserved; the number, however, was large.

The experiment confirms that the blue and indigo are the most efficient rays while the green are markedly less efficient. The yellow and red rays are inefficient. The longer exposure brings out the heliotropic effects in the extreme violet which do not show with shorter exposure.

In the following experiments an attempt was made to ascertain the influence of longer exposure. The first question was whether by making the duration of exposure considerably longer we should be able to induce heliotropic curvatures in the yellowish-green, yellow and red. Second, we wished to find out whether solarization effects might be observed in the case of too long an exposure.

The method was as follows: Different stems of *Eudendrium* with young polyps (prepared in the way described above) were put successively into the same limited part of the spectrum. Each stem was exposed a different length of time. The purpose was to find out how much time was required to cause the maximum number of polyps to bend toward the light (table 8).

TABLE 8.

*Experiment 7: Eudendrium Exposed to Light
of 4700 Å.u. (Blue).*

DURATION OF EX- POSURE IN MINUTES	FRACTION OF POLYPS BENT TO THE LIGHT	PERCENTAGE OF POLYPS BENT TO THE LIGHT
5	4/25	16
10	8/11	73
20	8/29	28
40	1/3	33
80	18/23	78
160	24/24	100

The experiments show that an exposure of more than ten minutes (for the light intensity used in our experiments) did not essentially increase the percentage of polyps bending to the light.

TABLE 9.

Eudendrium Exposed to Light Five and a Half Hours.

WAVE LENGTH IN ÅNGSTRÖM UNITS	COLOR OF THE SPECTRAL REGION	FRACTION OF POLYPS BENT TO THE LIGHT	PERCENTAGE OF POLYPS BENT TO THE LIGHT
4800	blue	7/16	44
4950	blue	1/6	16
5300-5500	green	3/13	24
5720	yellow	0/21	0
6000-6550	orange and red	0/32	0

We made with the same material an experiment in which the polyps were exposed for five and a half hours to the spectrum from blue to red with the result shown in table 9.

The experiment shows again, first, that even in five and a half hours the rays from yellow to red are without any heliotropic effect. Second, that the efficiency of rays with wave length of 4800 and above is less than that of wave length 4700. It is possible but not certain that there are solarization effects.

In the next series of experiments a region in the neighborhood of 5600 Å.u. in the yellow towards the yellowish-green was selected for the stems (table 10).

TABLE 10.

Experiment 8: Eudendrium Exposed Various Lengths of Time to the Same Wave Length (About 5600 Å.u.).

DURATION OF EXPOSURE IN MINUTES	POLYPS BENT TO THE LIGHT
10	0
20	0
40	0
80	0
160	0

In order to make sure that this negative result was not the fault of the material experiments with the same lot of material were carried out in the blue-violet part of the spectrum (table 11).

This experiment again shows strikingly that the blue and violet part of the spectrum is the effective one. The region between 4700 and 4750 Å.u. is again the most efficient.

TABLE II.

Eudendrium Exposed for One Hundred and Fifty Minutes.

WAVE LENGTH IN ÅNGSTRÖM UNITS	COLOR OF THE SPECTRAL REGION	FRACTION OF POLYPS BENT TO THE LIGHT	PERCENTAGE OF POLYPS BENT TO THE LIGHT
4220-4500	violet and indigo	21/24	87
4500-4660	indigo-blue	9/11	82
4660-4710	blue	7/10	70
4710-4750	blue	15/15	100
4750-4850	blue	14/16	85
4850-5000	blue-green	7/8	87

In a third experiment of this series the stems were exposed to a wave length of about 4900 Å.u. (blue towards the green) for various periods of time with the result shown in table 12.

TABLE 12.

Experiment 9: Eudendrium Exposed to Light of 4820 Å.u. (Blue).

DURATION OF EXPOSURE IN MINUTES	FRACTION OF POLYPS BENT TO THE LIGHT	PERCENTAGE OF POLYPS BENT TO THE LIGHT
0	2/9	22
10	5/11	45
20	8/12	75
40	7/11	64
80	11/13	73
160	5/18	28

With the same material an experiment with long exposure (five and a half hours) in the region of shorter wave lengths, 3750 to 4700 Å.u. (namely, from the blue to extreme violet) was carried out. In all, 52 hydroids out of 66 (i.e., 79 per cent) were bent forward. There was not much difference in the various regions, probably due to the long exposure.

III. CONCLUSION AND SUMMARY OF RESULTS.

These experiments have shown that the most efficient region in the spectrum for the production of heliotropic curvatures in the hydroid *Eudendrium* is situated in the blue at $\lambda \sim 4735$ Å.u. This region coincides approximately with the one found by Blaauw for the seedlings of oats namely $\lambda \sim 4780$ Å.u.

The regions in the red, orange and yellow are practically without effect in both *Eudendrium* and *Avena*.

The heliotropism of the sessile animal *Eudendrium* and that of the sessile plant *Avena* are therefore identical even as regards the most efficient wave length.

We expect to discuss the effects of different wave lengths upon the heliotropic reactions of motile animals and plants in another paper.

THE BLINDNESS OF THE CAVE FAUNA AND THE
ARTIFICIAL PRODUCTION OF BLIND FISH
EMBRYOS BY HETEROGENEOUS HY-
BRIDIZATION AND BY LOW
TEMPERATURES.

(WITH 13 FIGURES.)

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

I.

While many of the animals inhabiting caves are blind or have degenerated eyes, the same phenomenon is rarely found among animals that live in the open. At first sight this seems to suggest that the disuse of the eyes in the complete darkness of the cave has gradually led to the degeneration of the eyes and this idea seems at one time to have been widely accepted. In forming a judgment of the connection between the darkness of the caves and the blindness of cave dwellers we must remember that some of the cave dwellers have perfectly normal eyes. Thus Eigenmann, to whom we owe the most thorough study of this subject, points out that of the four species of salamanders living habitually in North American caves two have apparently quite normal eyes. They are *Spelerpes maculicauda* and *Spelerpes stejnegeri*. Two others living in caves have quite degenerate eyes, *Typhlotriton spelæus* and *Typhlomolge rathbuni*.¹ If disuse is the direct cause of blindness we must inquire why *Spelerpes* is not blind.

Another difficulty arises from the fact that a blind fish, *Typhlogobius*, is found in the open (on the coast of southern California) in shallow water, where it lives under rocks in holes occupied by

¹ Eigenmann, "Cave Vertebrates of America," Carnegie Institution Publications, Washington, 1909.

shrimps. One must again ask the question: How can it happen that in spite of the exposure to light *Typhlogobius* is blind?

The most important fact is perhaps the one found by Eigenmann in the fishes of the family of Amblyopsidae. Six species of this group live permanently in caves and are not found in the open, while one lives permanently in the open and is never found in caves and one comes from subterranean springs. The one form which is only found in the open, *Chologaster cornutus*, has a simplified retina aside from having a comparatively small eye, in other words, its eye is not normal. This indicates the possibility that the other representatives which are only found in caves also might have abnormal eyes even if they never had lived in caves.

Through these facts the old idea becomes doubtful, namely, that the cave animals had originally been animals with normal eyes in which the disuse had led to a gradual hereditary degeneration of the eyes. Instead we must consider the possibility that in the blind cave animals as well as in the blind animals which live in the open the tendency towards blindness developed independently of presence or absence of light. From this point of view the tendency towards degeneration of the eye appears as a hereditary mutation comparable to the inherited glaucoma which is known in the human. Glaucoma is a form of blindness caused by the atrophy of the optic nerve in consequence of an increased intraocular pressure and this high pressure seems to be caused by a certain disturbance in the circulation in the eye. The fact that these patients are born with normal eyes and do not become blind until later in life shows that lack of light has no share in the development of this hereditary disease or mutation. The question then remains, how can we account under such an assumption for the fact that blind animals are so prevalent in caves and so rare in the open? We shall return to this question later on.

II.

We will now review briefly the literature dealing with observations and experiments on the influence of light on the formation of eyes. The fact that the eyes of mammals are formed in complete darkness (in the uterus) may serve at the outset as a warning against overestimating the effect of light on the formation of eyes. F.

Payne¹ has raised sixty-nine generations of a fly (*Drosophila*) in complete darkness without noticing any changes in their eyes or in their sensitiveness to light.

Recently Uhlenhuth² has demonstrated in a very striking way that the development of the eye does not depend upon the influence of the light or upon its functioning. He transplanted the eyes of young salamanders into different parts of their bodies where they were no longer connected with the optic nerves. The eyes after transplantation underwent a degeneration which was followed by a complete regeneration. Uhlenhuth showed that this regeneration

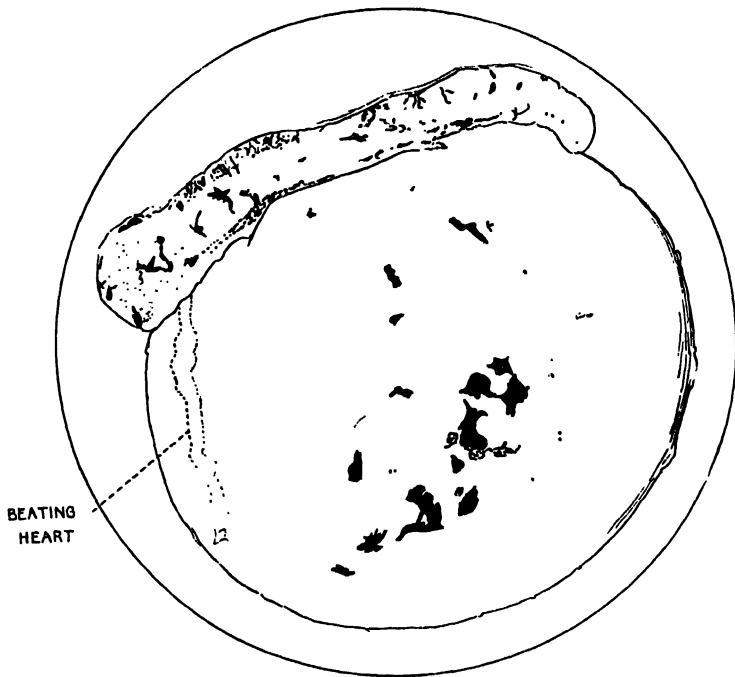


FIG. 1.

took place in complete darkness and that in salamanders, kept in the dark for 15 months, the transplanted eyes remained normal. Here the eyes were no longer in connection with the central nervous system, received no light, and could not possibly have functioned and

¹ F. Payne, *Biol. Bull.*, XXI., p. 297, 1911.

² Read at the meeting of Anatomists at St. Louis, December, 1914.

yet they regenerated and kept normal. The degeneration which took place in the eyes immediately after they were transplanted was apparently due to the interruption of the circulation in the eye and the regeneration probably set in with the reestablishment of the circulation in the transplanted organ.

The older literature had many observations which were assumed to prove an influence of light on development but the writer has shown in a former paper that these conclusions were not supported by the facts on which they rested.

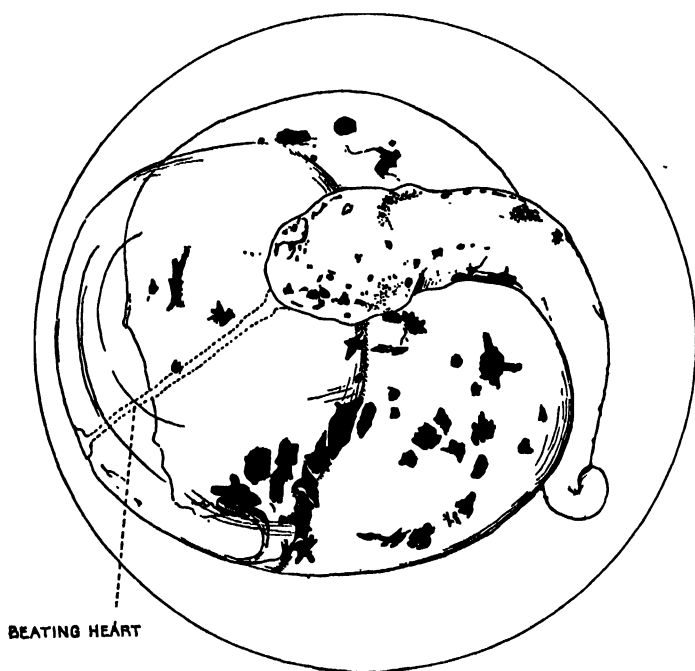


FIG. 2.

The writer has for years paid special attention to the possibility that light might influence the formation of organs in animals, but he has succeeded in finding only one form in which such an influence can with certainty be demonstrated, namely the hydroid *Eudendrium*. In his experiments in Naples he had noticed that the polyps of this form regenerate better in the light than in the dark and in Woods

Hole he could convince himself that the stems of *Eudendrium* can not regenerate their polyps if kept permanently in the dark.¹

Kammerer² seems to be the only author who still takes it for granted that cave animals owe the degeneration of their eyes to lack of light, and his support for this view consists in the statement that five young cave salamanders (*Proteus*) developed larger eyes under certain (somewhat puzzling) conditions of illumination. In other

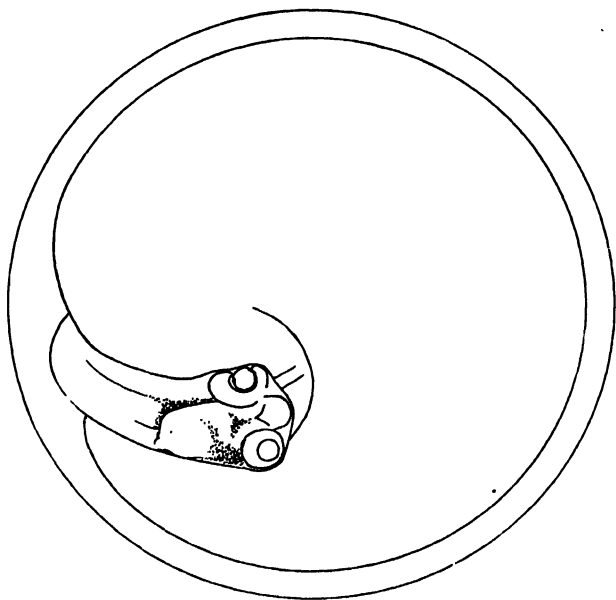


FIG. 3.

cases the eyes of *Proteus* remained unaltered in the light. It would be advisable to make certain whether or not there are two varieties of *Proteus* and moreover it would be desirable to repeat these experiments on a larger scale.

The writer wishes to publish in this note the results of some experiments made in 1912, which prove that in the fish *Fundulus* it is comparatively easy to produce embryos with degenerated eyes

¹ Loeb, "Über den Einfluss des Lichtes auf die Organbildung bei Tieren," *Arch. f. d. ges. Physiol.*, LXIII., p. 273, 1896; Goldfarb, *Jour. Exper. Zool.*, 3, 129, 1906; 8, 133, 1910.

² Kammerer, *Arch. f. Entwicklungsmechn. d. Organ.*, XXXIII., p. 350, 1912.

by various means except by the one Kammerer holds responsible for this phenomenon, namely lack of light. The reader may in passing be reminded of the fact that in this form Stockard and later McClendon induced the formation of cyclopean eyes by altering the constitution of the sea water (addition of magnesium salts or of alcohols and other narcotics).¹ The writer's attention to an abnormal development of the eyes in this embryo was first attracted in

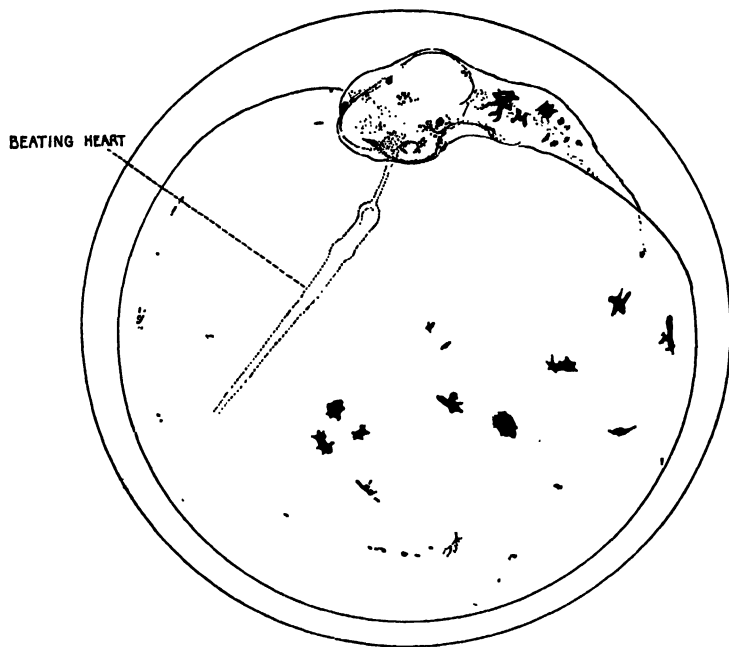


FIG. 4.

his experiments on heterogeneous hybridization.² He noticed that among the anomalies noticeable in heterogeneous fish hybrids the lack of circulation was the most common, but that incomplete or abnormal development of the eyes was not infrequent. Such embryos often give the impression that they have no eyes at all, though in reality a histological analysis would probably show that some of the

¹ Stockard, *Am. Jour. of Anatomy*, 10, 369, 1910; *Jour. Exper. Zool.*, 4, 165, 1907; 6, 286, 1909; McClendon, *Am. Jour. Physiol.*, 29, 289, 1912.

² Loeb, "Heredity in Heterogeneous Hybrids," *Jour. Morphol.*, XXIII., p. I, 1912.

organs of the eye are present. But it is safe to say that the condition of the eyes is such as to make them unfit to form a retina image and for this reason we may call these embryos with abnormal (or apparently lacking) eyes blind; in the same sense in which this term is used in the case of the blind salamanders or fish found in caves which also have some of the elements of an eye but in a condition unfit for use. The term "blind fish" is therefore used in the following as a synonym with imperfect or degenerated eyes.

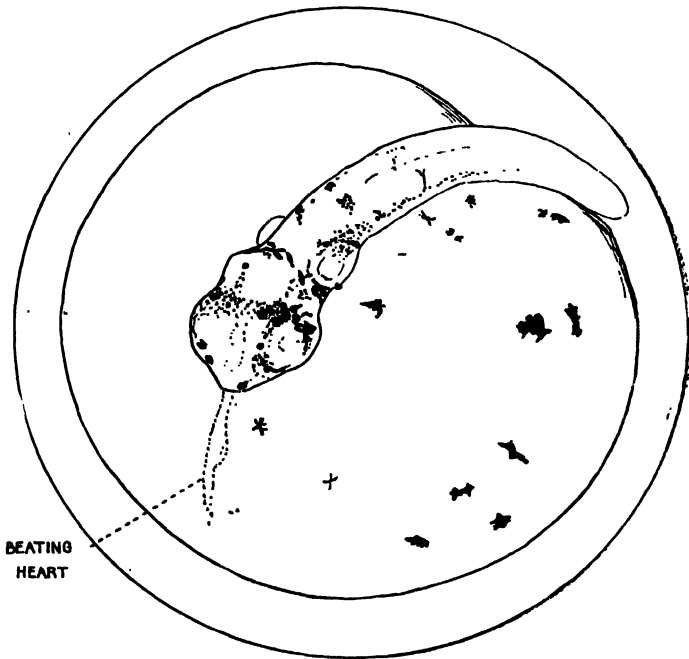


FIG. 5.

III.

Blindness Produced through Heterogeneous Crossings.

Figs. 1 and 2 are camera drawings of hybrids between *Fundulus heteroclitus* ♀ and *Menidia* ♂. The first embryo is eight, the second fourteen days old. In neither of these two embryos are eyes noticeable, though of course a histological examination might have divulged rudimentary eyes. The embryos are in this respect to all appearances comparable to the blind cave fish or salamanders.

It is a known fact that certain blind animals have in an early stage a comparatively better developed eye than in later stages, *i. e.*, that the eye either stops developing early while the development of the rest of the body continues or that the eyes degenerate directly. The writer noticed not infrequently that the eyes of the cross between *Fundulus* and *Menidia* were normal in the beginning but appeared more abnormal the further the development progressed. We will illustrate this statement by two series of drawings. The first case is illustrated by Figs. 3 to 6. Fig. 3 shows the embryo four days old. The embryo has normally developed eyes with lenses. The

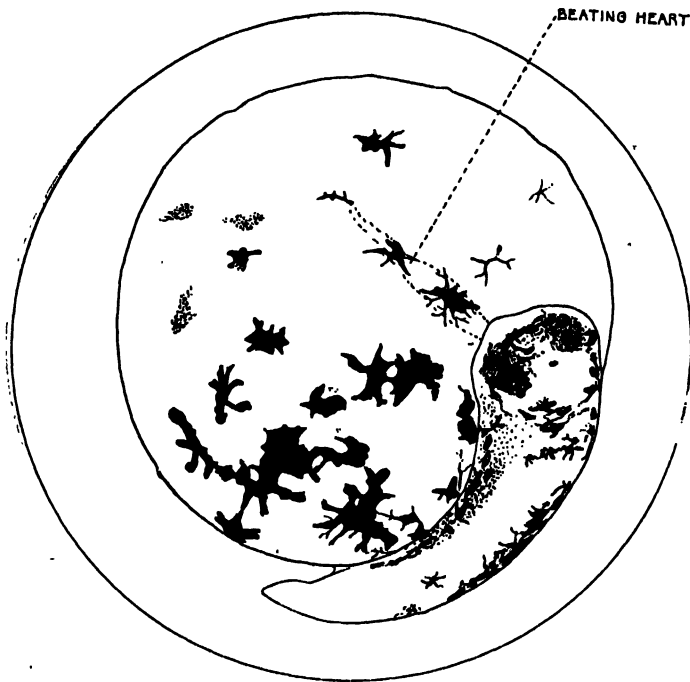


FIG. 6.

embryo's heart, which is visible in front, was not yet beating and no pigment was formed. The same embryo is shown two days later in Fig. 4. The eyes instead of developing any further are less distinct. Fig. 5 is the same embryo two days later (eight days old). The eyes are hardly recognizable, it is difficult to decide without sectioning

whether the lens still exists. The internal ear is distinguishable. The heart is pulsating.

Fig. 6 shows the same embryo at the age of two weeks. Apparently the pigment is the only organ of the eye which is developed.

Figs. 7 and 8 illustrate the same fact, that a rather normal eye with a lens was formed while later on only a very imperfect eye remained. Fig. 7 shows the embryo when four days old with a lens and an apparently normal eye, while five days later (Fig. 8) no eye was perceptible.

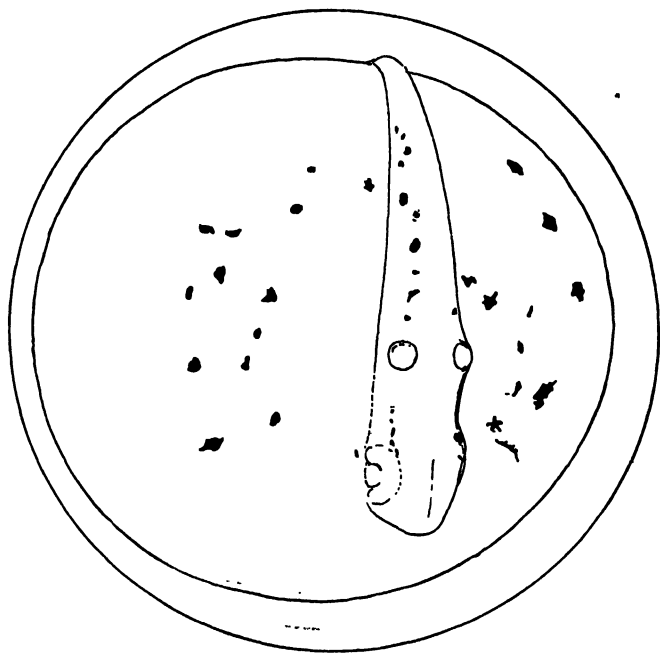


FIG. 7.

IV.

Blind Fish Produced from Pure Breeds of Fundulus Heteroclitus.

The writer was under the impression that the abnormalities in the development of the heterogeneous hybrids in fish were due to the influence of the foreign sperm as in the similar case of the fertilization of the egg of the sea urchin with the sperm of the star fish, where the mortality is also enormous. In order to prove this as-

sumption he tried to find a method which would allow him to produce embryos with abnormal eyes in the pure breeds of *heteroclitus*. He has already mentioned in a former publication that such abnormal embryos were obtained when their development was retarded by putting them into sea water to which some KCN was added. Fig. 9 shows a blind embryo obtained in this way. The eggs were put immediately after fertilization into a weaker solution of KCN in sea water where they remained sixteen days. They developed slowly.

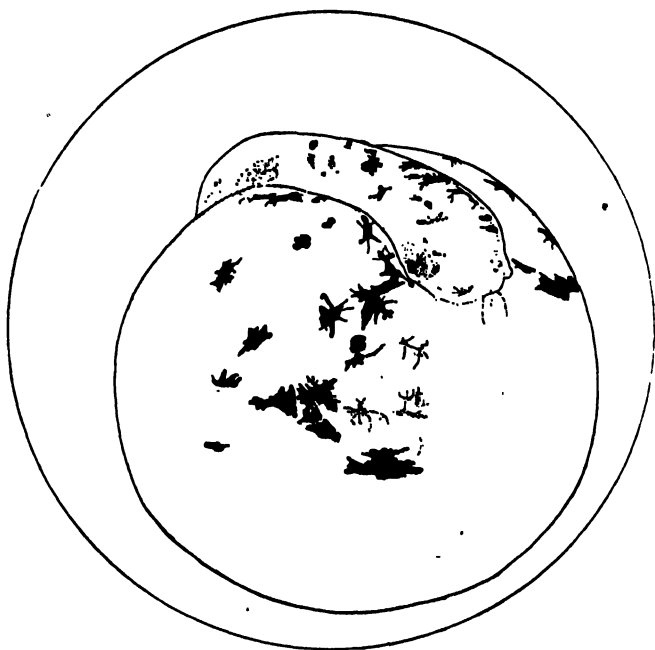


FIG. 8.

The embryo was drawn two days after it had been put back into normal sea water. The heart-beat and circulation were established. This method of producing blind embryos is not reliable and need not be discussed any further.

A better method was found by exposing the newly fertilized eggs to a low temperature (from between 0 to 2° C.) for some time. The writer found that the egg of *Fundulus* can be put for weeks into such a low temperature after the embryo is once formed, with-

out any injury to the latter. As soon as it is put back to room temperature it continues to develop. This corresponds with the idea that the low temperature only retards the chemical reactions underlying development. If, however, the egg of the same fish is exposed to such a low temperature immediately after fertilization or when the eggs are in the process of early segmentation they suffer severely.

Thus in one experiment the eggs of *Fundulus* (fertilized with sperm of their own species) were distributed into a number of Erlen-

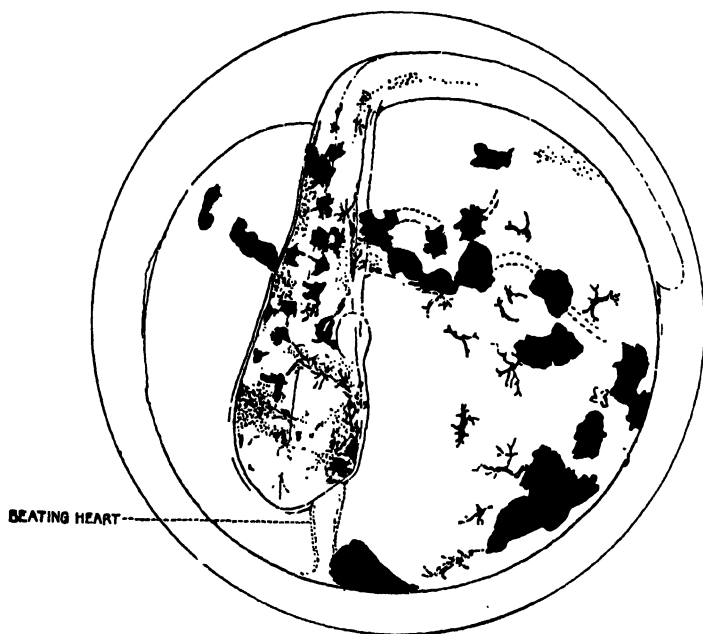


FIG. 9.

meyer flasks and put on ice immediately after fertilization and kept at a temperature of between 0 and 2° C. After four hours, seven and a half hours, twenty-three hours, thirty-two hours, forty-eight hours, sixty-four, and ninety-six hours one lot was returned to room temperature. All the eggs taken out after forty-eight hours or later were dead; few of those taken out after thirty-two and twenty-three hours survived. A considerable percentage were ab-

normal and resembled the heterogeneous hybrids between *heteroclitus* ♀ and *Menidia* ♂. Most of those taken out after four and seven and a half hours survived, but about 20 per cent. to 30 per cent. were abnormal. Among the abnormal embryos a number of blind fish were found.

A second lot of eggs were put on ice four and a half hours after fertilization when they were in the 4-cell stage or beyond. These eggs were just as sensitive to the effect of the low temperature as those put on ice immediately after fertilization.

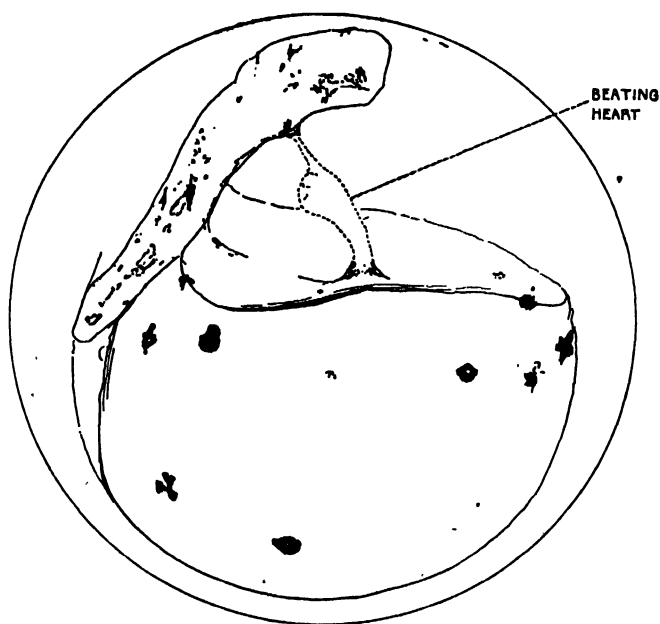


FIG. 10.

This experiment was repeated four times with the same result. In another experiment the eggs were allowed to develop to about the 128-cell stage at normal temperature before they were put into a temperature of 0° (about fifteen hours after fertilization). Eggs that were kept at that low temperature for two days were still able to develop into normal embryos but those that had been kept three days at the temperature of from 0 to 2° C. were practically all dead.

When the eggs were put into a temperature of from 0 to 2° C.,

after the embryo was formed and the circulation established, they could resist the low temperature for weeks. When put back to normal temperature they recovered and developed normally.

Further experiments are required to ascertain more accurately when the eggs become immune to the temperature of from 0° to 2° .

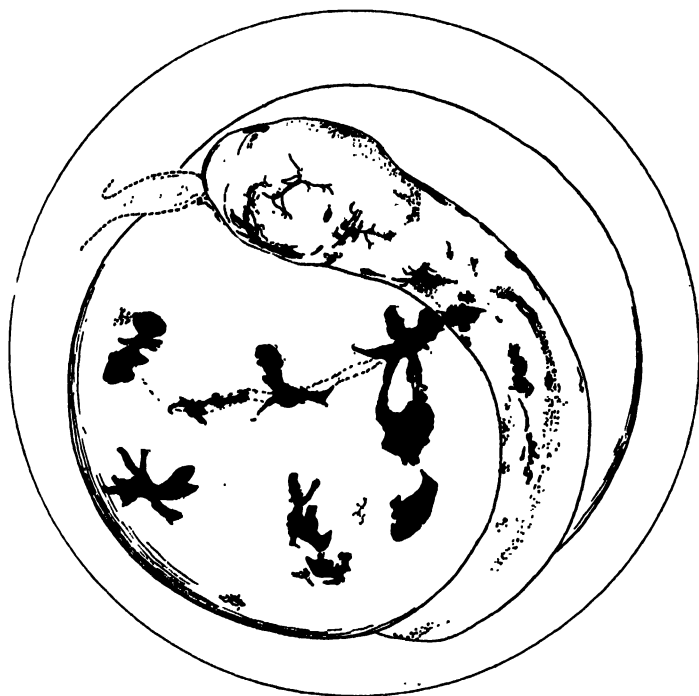


FIG. 11.

It is only this very low temperature at or very near the freezing point which is injurious for the newly fertilized eggs. If the temperature is a little higher, *e. g.*, 7° C., the newly fertilized eggs can live for weeks in it without being injured. Thus in the experiment just mentioned some of the eggs were put immediately after fertilization into a temperature of about 7° C. These eggs developed very slowly but no abnormal embryos were observed although some of the eggs were kept at a temperature of 7° C. for four weeks. Those that were kept still longer at that temperature suffered but

probably not from the low temperature but from the fact that the flasks in which they were kept were closed.

These experiments thus establish the interesting fact that immediately after fertilization the eggs of *Fundulus* are rapidly injured or killed when exposed to a temperature of 0° or a little above, while after the embryo is formed they can be exposed to such a temperature for a month without suffering. The newly fertilized eggs can, however, be exposed without injury to a temperature of 7° for weeks and possibly longer.

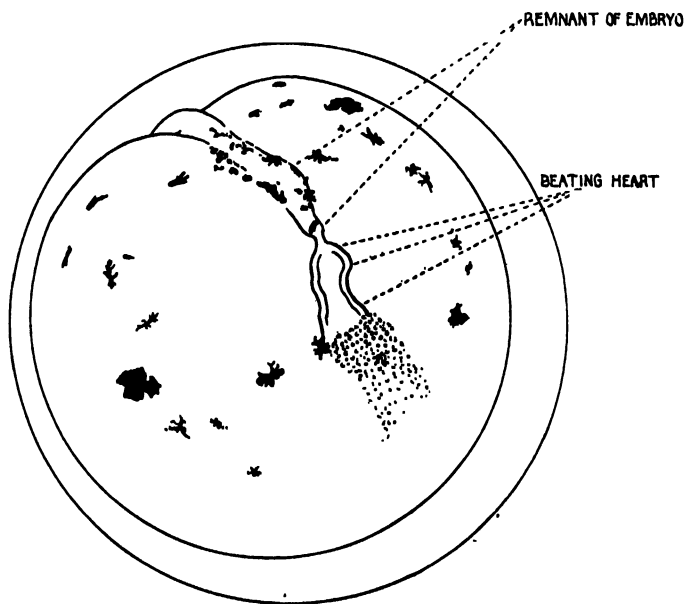


FIG. 12.

As already stated, among those eggs which were put into a temperature of from 0 to 2° C. a certain number had defective eyes. Thus the egg Fig. 10 was put immediately after fertilization into a temperature of between 0 and 2° for twenty-four hours and then put back to room temperature. The egg developed like a typical heterogeneous hybrid. Fig. 10 represents the embryo when two weeks old; it had a beating heart but no circulation. No eyes are noticeable. Fig. 11 represents an embryo from another experiment;

in this too the egg had been kept for four hours (immediately after fertilization) on ice (0 to 2°). Apparently no eyes are formed but the circulation is established.

Figs. 12 and 13 are added to show to what extent the abnormal embryos, produced by a short exposure of the egg to a very low temperature (0°) immediately after fertilization, resemble the monstrosities which are formed in the case of heterogeneous hybridization. The embryo in Fig. 12 consisted practically only in a pulsating heart, what was left of the embryo was much less conspicuous than it is in the drawing. The embryo was ten days old. The egg

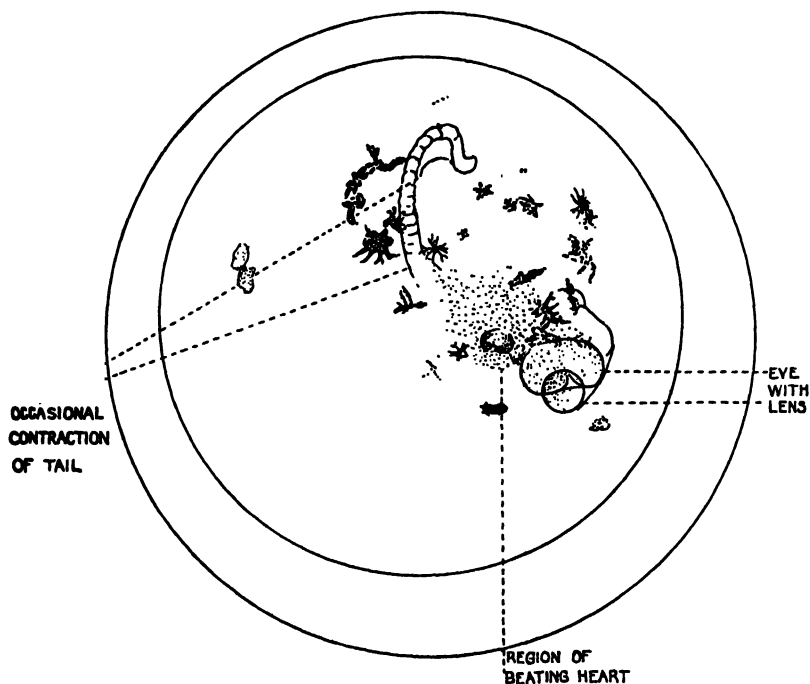


FIG. 13.

had been put into the temperature of between 0 and 2° when it was in the 2-cell stage and had remained there for twenty-four hours.

The remnants of the embryo Fig. 13 had the same history. In this case, curiously enough, one eye with a lens and the tail was all that could be recognized. The monstrosity when drawn was

eleven days old. An inexperienced observer might easily have concluded that in this case only the eye had been developed, while in reality the whole embryo had developed but the eye had survived while other parts perished.

The analogy with teratomata is obvious.

V.

The writer was anxious to know whether it was possible to produce deficiencies in the eyes of *Fundulus* by raising them in the dark. It was necessary to carry on such an experiment with a large number of embryos. Since it was possible that a short exposure of the unfertilized egg or of the sperm to the light might already have some effect, the females and males of *Fundulus heteroclitus* to be used for the experiment were put into an absolutely dark room where everything was prepared for the experiment. The females and males were stripped of their sexual cells in the dark and the jars containing both sperm and eggs were put into dark boxes and kept in the dark room for four weeks. Although many embryos had died, hundreds had survived. All had perfectly normal eyes. This experiment confirms similar experiments made by the writer in previous years.

All these experiments show that while it is comparatively easy to produce blind *Fundulus* embryos, or, more correctly speaking, fish with degenerated eyes, by heterogeneous hybridization or by low temperature or by lack of oxygen (or by an excess of magnesium salts or by alcohol, as shown by Stockard and McClendon) no such result can be produced by lack of light.

VI.

If we consider all the facts in the case there is nothing at present to warrant the assumption that the blind cave animals owe the deficient development of their eyes to the lack of light, since lack of light is according to our present knowledge a less efficient agency in the causation of an abnormal development of the eye than a number of other injurious influences. Under these conditions we must be prepared to consider the possibility that many if not all the blind

species found in caves owe their blindness to other influences than those of the cave. Eigenmann states that no blood vessels enter the eye of the blind cave salamander *Typhlotriton*. Since the experiments of Uhlenhuth as well as those of Stockard and of the writer reported in this paper indicate the importance of the circulation and of chemical factors in the development of the eyes it is not impossible that in the blind fish (Amblyopsidæ) as well as in the blind salamanders a hereditary disturbance in the circulation or nutrition of the eye or its surroundings is the cause of the degeneration of the eyes. There remains then the one difficulty mentioned in the beginning, namely to account for the fact that the relative number of blind species is greater in caves than in the open.

Eigenmann has shown that all those forms which live in caves were adapted to life in the dark before they entered the cave. These animals are all negatively heliotropic and positively stereotropic and with these tropisms they would be forced to enter the cave whenever they are put at the entrance to the cave. Even those among the Amblyopsidæ which live in the open have those tropisms of the cave dweller. This eliminates the idea that the cave adapted the animals for the life in the dark.

Eigenmann's observation makes it also clear that blind animals are comparatively rare in the open and that animals with normal eyes are not in the majority in the caves. Only those animals can thrive in the caves which for their feeding and mating do not depend upon visual mechanisms; and conversely animals which are not provided with visual mechanisms can only under exceptional conditions hold their own in the open where they meet the competition of animals which can see. This would account for the fact that in caves blind species are comparatively more prevalent than in the open.

In spite of all this, Eigenmann is inclined to assume that the darkness of the caves was a factor in promoting the blindness of the cave fauna. While those Amblyopsidæ which live in the open have already abnormal eyes those species of the family which are found in the caves have more degenerated eyes than those in the open and Eigenmann is inclined to ascribe this fact to an accumulated influence of the darkness. This would, however, compel

us to account in a similar way for the incipient degeneracy of the eyes of those Amblyopsidæ that have never been found in caves and for the complete blindness of *Typhlogobius*; and would leave us at a loss to account for the presence of salamanders with perfectly normal eyes in the caves. It seems to the writer that consistency would demand to consider a common mode of origin of all these blind forms, namely as mutations, *i. e.*, as the result of some factorial change in the germ the cause and nature of which we are not yet able to define. Among the Amblyopsidæ various mutations in the state of the eye may be constantly arising (as in the case of *Drosophila*); and some of these mutants, especially those that are perfectly blind, can not hold their own in the open while in the caves they can preserve and perpetuate themselves. This assumption would not exclude the possibility that Kammerer's observation may have been correctly interpreted by him, it would only provide for the possibility that his conclusion can not be generalized—a possibility which from the experiments mentioned in this paper must be seriously considered.

SUMMARY.

1. It is shown that blind embryos or more correctly embryos with degenerated eyes can be produced by heterogeneous hybridization in fish embryos (*e. g.*, *Fundulus heteroclitus* ♀ and *Menidia* ♂). Since in these cases as a rule no circulation exists the inference is possible that the anomalous condition of the eye may be due to lack of circulation.

2. Blind embryos of the pure breed of *Fundulus heteroclitus* may be produced by the addition of KCN to the sea water.

3. It is shown that immediately after fertilization (by sperm of their own species) and during the early stages of segmentation the egg of *Fundulus heteroclitus* is rapidly killed or injured if it is exposed to a constant temperature of about 0° (or slightly above); while it may be exposed to a slightly higher temperature (*e. g.*, 7° C.) for weeks without being injured. If the egg is exposed to the low temperature after the embryo is once formed it can resist the low temperature of from 0 to 2° C. for weeks without permanent injurious effects.

4. If eggs of *Fundulus heteroclitus* are fertilized with the sperm of the same species and exposed immediately after fertilization for a number of hours or a day to a temperature of between 0 and 2° C. abnormal embryos can be produced a certain percentage of which may show degenerated eyes.

5. Lack of light does not influence the development of the eyes of *Fundulus*.

6. It is pointed out that internal mutational changes and not lack of light may account for the blindness of certain cave fish and salamanders.

REVERSIBLE ACTIVATION AND INCOMPLETE MEM- BRANE FORMATION OF THE UNFERTILIZED EGGS OF THE SEA URCHIN.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

1. The writer showed in 1913 that the artificial activation of the egg can be reversed under certain conditions.¹ When the egg of *Arbacia* is treated with a base in the proper concentration and for the proper length of time the effect is somewhat similar to that which follows the treatment of the egg with a fatty acid. The egg is induced to develop, it may segment (as a rule abnormally) once or twice, and if nothing else happens it will perish rather rapidly (unless the developmental processes are prevented). If the eggs after the treatment with the acid or with the base receive a treatment with a hypertonic solution of the proper concentration and for the proper time they may develop into larvæ.

The writer found that if the eggs are treated with alkali in the proper way to induce development and if they are immediately afterwards put for several hours into a solution which prevents their development (sea-water with chloral hydrate or NaCN) the eggs when taken out behave as if nothing had been done to them. They neither segment nor do they disintegrate and they can again be induced to develop by fertilization (or probably with the methods of artificial parthenogenesis though the writer has not yet tried this). The activating effect of the alkali is therefore reversible.

This reversibility is, however, only possible if the eggs have not been exposed to the alkali too long. After too long an exposure the effect of the alkali is no longer reversible by a temporary suppression of the developmental processes. A second condition for the reversibility is that the eggs are immediately transferred from the

¹ Loeb, *Science*, N. S., XXXVIII., 749, 1913; *Arch. f. Entwicklungsmech.*, XXXVIII., 277, 1914.

alkaline solution into the chloral hydrate or NaCN solution. If they are first transferred into normal sea-water and then after fifteen or thirty minutes later are transferred to the cyanide solution nothing may happen to them as long as they remain in the cyanide solution (unless the HCN evaporates) but they will disintegrate when put back into normal sea-water. From this we must conclude that in the sea-water the processes started in the alkaline solution will continue and the egg behaves as if it had been overexposed to the hyperalkaline solution.

Somewhat similar experiments were made with the eggs of *Arbacia* which had been treated with butyric acid. When such eggs were put over night into the cyanide-sea-water immediately after the artificial membrane formation they did not as a rule disintegrate when put back into normal sea-water but could be fertilized afterwards. The writer is, however, not certain that the phenomenon of reversibility is as constant here as in the case of the alkali treatment.

The writer pointed out that the explanation for this phenomenon could probably be found if we compare the behavior of the eggs of *Arbacia* with that of eggs of *purpuratus* under similar conditions. If we cause the production of a butyric acid membrane in the egg of *purpuratus* the activation of the egg is usually irreversible. In the egg of *purpuratus* the membrane which is formed under the butyric acid treatment is very tough and is separated by a wide area from the protoplasm, while in the egg of *Arbacia* the membrane consists often only of a fine gelatinous film which lies tightly around the egg. The writer concluded from this that there may be a quantitative if not a qualitative difference in the amount of change produced by the butyric acid treatment in the cortical layer of the two kinds of eggs; in the egg of *Arbacia* where this change is quantitatively smaller the activation is reversible, while in the egg of *purpuratus* where the change is quantitatively larger (and possibly also qualitatively different) it is irreversible (Loeb, "Artificial Parthenogenesis and Fertilization," Chicago, 1913, p. 286).

2. This idea is further supported by the curious phenomena of reversibility in the eggs of *Strongylocentrotus purpuratus*. When one tries to induce artificial parthenogenesis in the eggs of *purpuratus* by a treatment with a hypertonic solution (without artificial mem-

brane formation) it is found that this is possible only with the eggs of certain females. Such eggs develop into plutei. In the eggs of other females a small percentage of eggs will begin to segment and they may go to the 2, 4, 8, or 16-cell stage or still a little further but then stop developing. Such eggs will go into the resting stage again and are normal to all external appearances. If fertilized with sperm a number of hours or a day later the individual blastomeres will each form a special fertilization membrane and develop into small but apparently perfect larvæ.¹ This experiment shows that the activating effect which the hypertonic solution had was reversed, since all these eggs were at first in the state of active development.²

3. The writer wishes to report in this paper the fact that in the egg of *purpuratus* under definite conditions a membrane formation can be produced by butyric acid which leads neither to any development if followed by the usual treatment with a hypertonic solution nor to a rapid disintegration when not followed by any "corrective" treatment. Since in all previous observations on the effects of membrane formation by butyric acid the reverse was found it seemed of theoretical importance to study this exception.

The main condition for the experiment is that the eggs are put after the treatment with butyric acid into a $m/2$ solution of $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ instead of into sea-water; the various salts are always used in the proportion in which they are contained in sea-water. The eggs were first washed three times in a mixture of $m/2$ $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ and were then put for from one and a half to two and a half minutes into a mixture of 50 c.c. $m/2$ $\text{NaCl} + \text{KCl} + \text{CaCl}_2 + 1$ c.c. $N/10$ butyric acid; from here they were transferred into a neutral or a slightly alkaline solution of $m/2$ $\text{NaCl} + \text{KCl} + \text{CaCl}_2$. In this solution all the eggs form a membrane which is very thin and which is at first at a great distance from the egg. Figs. 1 and 2 give

¹ Loeb, *Arch. f. Entwicklungsmech.*, XXIII., 479, 1907; "Artificial Parthenogenesis and Fertilization," Chicago, 1913, p. 237.

² Only the activating effect of the hypertonic solution is reversible, the second, corrective effect, which the hypertonic solution imparts to the egg was not reversed since these blastomeres develop also if only the artificial membrane formation is induced in them by butyric acid, while without the previous treatment with a hypertonic solution such eggs would soon perish. Loeb, *Jour. Exper. Zool.*, XV., 201, 1913; "Artificial Parthenogenesis and Fertilization," p. 238.

an idea of the first appearance of this membrane; the membrane in Fig. 1 was formed in a neutral, that of Fig. 2 in a faintly alkaline solution. Later on, the membrane collapses and is found lying rather close to the egg. Such eggs can be readily fertilized with sperm in spite of the existence of the membrane. The latter is either naturally permeable for sperm or it is easily torn and thus allows the sperm to reach the egg or it is not entirely continuous around the eggs.

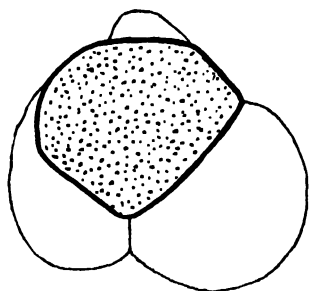


FIG. 1.

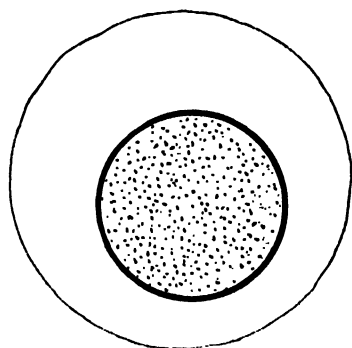


FIG. 2.

Eggs were washed three times in a mixture of $m/2$ $\text{NaCl} + \text{CaCl}_2 + \text{KCl}$ and then put for from one and a half to two and a half minutes into 50 c.c. $m/2$ $\text{NaCl} + \text{KCl} + \text{CaCl}_2 + 1.0$ c.c. $N/10$ butyric acid. From this solution they were transferred:

(a) Into normal sea-water.¹

(b) Into a neutral mixture of $m/2$ $\text{NaCl} + \text{KCl} + \text{CaCl}_2$.

Lot *a* formed normal fertilization membranes and disintegrated in a few hours. The eggs of lot *b* formed the fine fertilization membrane which was at first very distant from the egg. Some of these eggs were transferred into normal sea-water after one hour. These eggs did not form a better membrane but perished in the same way as the eggs of lot *a*.

A second portion of lot *b* was transferred into normal sea-water after having been for seven hours in the mixture of $m/2$ $\text{NaCl} +$

¹ Such eggs have at first a tendency to stick to the glass when transferred to sea-water. It is necessary to keep them in slight agitation for some time until they have lost their tendency to stick.

$KCl + CaCl_2$. These eggs did not disintegrate during the following night although they did not appear quite normal the next day. The rest of the eggs remained in the $m/2$ $NaCl + KCl + CaCl_2$ solution for twenty-four hours. When these eggs were put into sea-water they did not disintegrate. To some of these eggs sperm was added the next morning and about 20 per cent. developed.

This experiment indicates that the nature of the membrane and the cortical changes which activate the egg of *purpuratus* are to a large extent determined by the solution into which the eggs are put after the butyric acid treatment. If the eggs are put directly into sea-water after the butyric acid treatment they form the typical membrane and rapidly perish if nothing else happens to them. The same may be the case if they are transferred from the butyric acid solution into a neutral solution of $m/2$ $NaCl + KCl + CaCl_2$ for a short time (less than one hour) and if they are afterwards transferred into normal sea-water. But if they remain in the $NaCl + KCl + CaCl_2$ solution for a number of hours they will as a rule not disintegrate if put back into sea-water.

A second experiment may be quoted. Eggs (that had been washed three times in a neutral $m/2$ $NaCl + KCl + CaCl_2$ solution) were put for one and a half to two and a half minutes at a temperature of about 14° into 50 c.c. of the same solution + 1 c.c. $N/10$ butyric acid. From here the majority of the eggs were transferred into a neutral solution of $m/2$ $NaCl + KCl + CaCl_2$ and the rest into sea-water. The latter formed normal fertilization membranes and disintegrated the same day. Those transferred into the neutral $NaCl + KCl + CaCl_2$ solution had all formed membranes which were at first separated from the egg by a great distance. They were kept in this solution over night and the next morning they were transferred into normal sea-water. All looked normal except that the membrane now formed a fine veil lying closely to the egg. Some of these eggs were fertilized with sperm. They all segmented and developed into swimming larvæ. The others were kept in sea-water without sperm to find out whether they would now disintegrate or whether the effect of the butyric acid treatment had worn off. This was the case. The eggs no longer disintegrated in sea-water. A fraction of these eggs was fertilized later and segmented.

When the eggs are transferred into normal sea-water immediately after the butyric acid treatment they perish rapidly if no second treatment is given to them. If they are treated with hypertonic sea-water (50 c.c. sea-water + 8 c.c. $2\frac{1}{2}$ *m* NaCl or NaCl + KCl + CaCl₂) for from thirty to sixty minutes they will develop into larvæ. If they are treated longer they will begin to develop but form very abnormal larvæ.

If the eggs, however, after the butyric acid treatment are not put into sea-water but into a mixture of $m/2$ NaCl + KCl + CaCl₂ in which they form the thin membrane and if they are kept here until they no longer disintegrate in normal sea-water, they cannot be induced to develop when treated for from thirty to sixty minutes with a hypertonic solution. Such eggs have, therefore, in spite of their membrane formation gone back into the condition of a resting egg.

4. It was obvious from these experiments that the constituents of the sea-water determine the nature of the membrane after butyric acid treatment. The writer expected to find that Ca was an absolute prerequisite for the membrane formation but this was not the case, although the presence of CaCl₂ improves the character of the membrane. Eggs that had been taken out of a $m/2$ NaCl solution were treated with 50 c.c. $m/2$ NaCl + 1 c.c. *N/10* butyric acid for one and a half to two and a half minutes. They were then transferred into the following three sets of solutions:

- (1). NaCl + KCl,
- (2). NaCl + CaCl₂,
- (3). NaCl + KCl + CaCl₂,

which were made in duplicates, the one set remaining neutral, the second set faintly alkaline (0.5 c.c. *N/100* NaOH were added to 100 c.c. of the solution). The result was not very different in the neutral and alkaline solutions. Many but not all the eggs put into NaCl + KCl formed very thin membranes often only in part of the egg. The membrane formation does therefore not require the addition of CaCl₂, but the eggs transferred into NaCl + CaCl₂ or into NaCl + KCl + CaCl₂ had formed a better and apparently stronger membrane than those put into NaCl + KCl.

In another experiment it was found that in a mixture of NaCl + MgCl₂ in the proportions in which these two salts occur in the sea-

water a membrane formation was also possible, but the membrane had the same fine and thin character. In all these cases the eggs could be fertilized with sperm in spite of the membranes. The writer is under the impression that no second membrane was formed upon the addition of sperm and only the hyaline membrane developed later. That the eggs after this inefficient membrane formation were not in the same condition as eggs which are put into normal sea-water after artificial membrane formation was shown by the fact that when put into a hypertonic solution for from thirty to sixty minutes not a single egg could be induced to segment.

The fact that the membrane always collapsed after some time seems to indicate that it soon became permeable for the colloids liquefied at the surface of the egg in the process of membrane formation.

5. The explanation of the phenomenon of ineffective membrane formation will probably be the same as that for the cases of reversible activation of the egg. It is an established fact that the rate of oxidations in the egg of *purpuratus* is raised about 600 per cent. by the act of fertilization. This rise is entirely due to the alteration of the surface layer of the egg which results in the membrane formation since artificial membrane formation alone has the same effect on oxidation. Even if the egg is killed in the process of membrane formation, *e. g.*, if we treat the unfertilized egg with saponin and leave it in this solution, the rate of oxidation is raised to the same amount.¹ The amount to which the artificial membrane formation raises the rate of oxidations in a sea urchin egg seems to be a constant for each species and not to depend upon the nature of the agencies employed for this purpose.

When we treat the unfertilized eggs of *purpuratus* with a hypertonic solution (without inducing a membrane formation) the eggs of some females will develop into larvæ, while the eggs of others will not. The writer assumed that the hypertonic solution if applied to the unfertilized egg has two effects, one consisting in the alteration of the surface layer (comparable to the effect of butyric acid) and secondly the corrective effect. Wasteneys and the writer² published a series of measurements on the influence of a hypertonic

¹ Loeb and Wasteneys, *Jour. Biol. Chem.*, XIV., 479, 1913.

² Loeb and Wasteneys, *Jour. Biol. Chem.*, XIV., 474, 1913.

solution on the rate of oxidations in unfertilized eggs of *purpuratus*, and found that the effect varied enormously with the eggs of various females. The eggs were in the hypertonic solution for one hour and the rate of increase in oxidation varied for the eggs of various females between 40 and 400 per cent. As we stated above, the eggs of only a limited number of *purpuratus* can be induced to develop into larvæ by a mere treatment with a hypertonic solution and it is probable that the eggs in which the oxidations were raised efficiently were the ones that could be induced to develop into larvæ in this way, while those in which the rate of oxidations was not raised to the same height did not segment. We also noticed that in the latter type of eggs the rate of oxidations diminishes after some time. It is possible that the reversion of development in such cases is due to a decline in the rate of oxidations below the height required for development.

The same possibility holds for the lack of development of the eggs after artificial membrane formation through butyric acid when they are afterwards put into a solution of $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ (instead of into sea-water). The fact that no correct membrane is formed and that the eggs neither develop nor disintegrate, and behave towards a treatment with the hypertonic solution like eggs without a membrane, arouses the suspicion that in this case the butyric acid treatment did not lead to the proper increase in the rate of oxidations. It is intended to investigate this possibility next summer, since it may also furnish the explanation of the phenomena of reversibility in development.

THE OCULOCARDIAC REFLEX.

AN ELECTROCARDIOGRAPHIC STUDY WITH SPECIAL REFERENCE TO THE DIFFERENCES BETWEEN RIGHT AND LEFT VAGAL AND OCULAR PRESSURES IN TABETICS AND NON-TABETICS.*

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Introduction and Historical Review.

In 1908 B. Aschner¹ first observed that pressure on the eyeball caused slowing of the pulse and decrease in the depth of respirations. He found that it would arouse stuporous, anesthetized and unconscious patients, and enable them to respond to questions. He also showed that in narcosis the oculocardiac reflex lasts longer than the corneal or pupillary reflexes. Grossmann and Miloslavich² in 1912 made similar observations, and Fabre and Petzetakis,³ working six years later than Aschner, confirmed the observation that the oculocardiac reflex persists even under deep ether or chloroform anesthesia, and can be elicited after the corneal reflex has gone. It was demonstrated by Aschner that the afferent impulse of the reflex passes through the trigeminal nerve to its nucleus in the midbrain, and that the efferent passes by way of the pneumogastric nerve. By cutting the third, fourth, sixth, seventh or eighth cranial nerves he could not destroy the reflex, but by cutting the fifth the reflex was

* This work was done in part under a grant from the Proctor Fund of the Harvard Medical School for the study of chronic diseases.

1. Aschner, B.: Ueber einen bisher noch nicht beschriebenen Reflex vom Auge auf Kreislauf und Atmung, Wien. klin. Wchnschr., 1908, xlv, 1529.

2. Grossmann, J., and Miloslavich, E.: Ueber die Beeinflussung der Herz-tätigkeit durch Bulbusdruck, Wien. klin. Rundschau, 1912, xii, 177.

3. Fabre and Petzetakis: Persistance du réflexe oculo-cardiaque pendant l'anesthésie générale, Compt. rend. Soc. de biol., 1914, lxxvi, 343.

destroyed. He also showed that the inhibition of the heart was not due to increased intracranial pressure, for the relief of pressure by trephining the skull did not abolish the reflex.

Milian,⁴ overlooking Aschner's discovery of five years before, stated in 1913 that the phenomenon of pulse-slowng resulting from ocular pressure should be called "*la signe de Gautrelet*" after one of his pupils who had been studying this reflex in his hospital for two years. This would bring back Gautrelet's first observation to three years after Aschner's discovery. Milian found that one could obtain the normal oculocardiac reflex in a dog breathing normally, whether anesthetized or not. But if the thorax were opened and artificial respiration instituted, no slowing of the pulse followed ocular pressure, but instead the tone of the ventricles was increased. This is the experimental evidence for the existence of the reflex and the basis of its mechanism. Many reports dealing with it from a clinical aspect have now been published.

Loeper and Mougeot⁵ investigated the condition of the oculocardiac reflex in certain varieties of gastric neurosis, namely, hypervagotonic and the hypersympathicotonic. The former are recognized by the presence of some of the following symptoms: paleness of the face, tendency to myopia, bradycardia, low blood-pressure, moist skin, asthma, gastric hypersecretion, hyperchlorhydria, rapid gastric motility, spasmodic constipation, etc. The latter present the opposite set of symptoms. The former react to pilocarpin with sweating and salivation, but do not react to epinephrin. The latter do not react to pilocarpin, but react to epinephrin with tachycardia, hypertension and glycosuria. They found that the oculocardiac reflex was exaggerated in the hypervagotonic cases and diminished or absent in the hypersympathicotonic. Similar results were obtained in an elaborate investigation by Gautrelet.⁶ In several instances they claim to have accelerated the pulse rate by ocular pressure, and they explain this unusual result by stating that the reflex which is gener-

4. Milian: Du ralentissement du pouls radial au cours de la compression oculaire dans la maladie de Basedow, Bull. et mém. Soc. méd. d. hôp. de Paris, 1913, No. 14, p. 878.

5. Loeper, M., and Mougeot, A.: Le réflexe oculo-cardiaque dans le diagnostic des nevroses gastriques, Bull. et mém. Soc. méd. d. hôp. de Paris, 1913, No. 14, p. 865.

6. Gautrelet, J.: Le réflexe oculo-cardiaque, Paris méd., Nov. 29, 1913, p. 583.

ally conveyed by the vagus at times passes over the sympathetic nerve. They consider that an individual whose pulse rate is accelerated or slowed more than ten beats per minute by pressure is abnormal, in the sense that he belongs to one or the other of the two groups named. This is a point to which reference will be made again later.

Lesieur, Vernet, and Petzetakis^{7, 8, 9} have studied the condition of the oculocardiac reflex in epileptics. This reflex, as well as tendon and cutaneous reflexes, was found to be exaggerated. In their experience it was found that bromid treatment not only lessened the frequency of epileptic attacks, but also diminished the oculocardiac reflex by decreasing vagal irritability. In the same study they found that the right eye responded more strikingly and on slighter pressure than did the left. Usually, when atropin was injected subcutaneously, it abolished the oculocardiac reflex, but ocular pressure continued to inhibit the respiratory motions of the patient just as it did when no atropin was given. This they called the oculophrenic reflex. Later, the same authors¹⁰ found that ocular pressure caused glycosuria in three of their six cases of epilepsy. Four showed albuminuria and all had polyuria. This reflex, they presumed, traveled over the sympathetic nerve and not the vagus, because in Claude Bernard's experiments in which he punctured the floor of the fourth ventricle, he observed glycosuria in animals when the vagi were divided, but not when all the sympathetic fibers were cut.

Guillain and Dubois,¹¹ in a case of double athetosis, noticed that the athetoid movements of the face, tongue and extremities ceased

7. Lesieur, Ch.: Vernet, M., and Petzetakis: Sur un cas d'arrêt total du cœur par réflexe oculo-cardiaque chez un épileptique, Bull. et mém. Soc. méd. d. hôp. de Paris, 1914, No. 9, p. 394.

8. Lesieur, Ch., Vernet, M., and Petzetakis: Contribution à l'étude du réflexe oculo-cardiaque. Son exagération dans l'épilepsie, les variations sous l'influence d'actions médicamenteuses ou toxiques, Bull. et mém. Soc. méd. d. hôp. de Paris, 1914, No. 9, p. 440.

9. Lesieur, Ch., Vernet, M., and Petzetakis: Considerations sur les modifications des réflexes pendant la compression oculaire chez certains épileptiques, Bull. et mém. Soc. méd. d. hôp. de Paris, 1914, No. 11, p. 510.

10. Lesieur, Ch., Vernet, M., and Petzetakis: Glycosurie, albuminurie, polyurie provoquées par la compression oculaire, Bull. et mém. Soc. méd. d. hôp. de Paris, 1914, No. 11, p. 515.

11. Guillain, G., and Dubois, J.: Action inhibitrice de la compression oculaire sur les mouvements anormaux dans un cas d'athetose double, Bull. et mém. Soc. méd. d. hôp. de Paris, 1914, No. 16, p. 850.

during ocular pressure, but increased during any other medical manipulation. Obstinate yawning and hiccough were observed by Loeper and Mlle. Weil¹² to be influenced favorably by ocular pressure. In six cases of pseudobulbar paralysis, Guillain and Dubois¹³ found that four had lost the oculocardiac reflex, and that the other two responded by an acceleration of the pulse rate. The condition of the oculocardiac reflex in various tremors has been studied by Lesieur, Vernet and Petzetakis.¹⁴ Of sixteen cases of Parkinson's disease the reflex was absent in fifteen. Of four cases of multiple sclerosis the reflex was absent in one, accelerated in two and normal in one. The reflex was normal in two cases of alcoholic tremor and in two cases of senile tremor. Of six cases of general paresis the reflex was exaggerated in four and normal in two. They concluded that the oculocardiac reflex might aid in determining whether a nervous lesion was central or peripheral. It does not seem clear, however, how the differentiation is to be made, for a reflex may be either exaggerated or destroyed by a lesion of the afferent limb, center, or efferent limb of a reflex arc. The condition alone of a reflex will scarcely determine the site of a lesion.

In the study of the reflex in organic diseases of the central nervous system, Miloslavich¹⁵ in 1910 was the first to observe that the reflex was absent in tabes dorsalis. Loeper and Mougeot^{16, 17} and Lesieur, Vernet and Petzetakis¹⁸ have shown that in a majority of such

12. Loeper, M., and Mlle. Weil: Action favorable de la compression oculaire sur certaines manifestations nerveuses et en particulier sur le hoquet, *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1914, No. 13, p. 631.

13. Guillain, G., and Dubois, J.: L'abolition et l'inversion du réflexe oculo-cardiaque dans les paralysies pseudo-bulbaires, *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1914, No. 12, p. 584.

14. Lesieur, Ch.: Vernet, M., and Petzetakis: Le réflexe oculo-cardiaque chez les sujets atteints de divers tremblements, *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1914, No. 12, p. 593; Réflexe oculo-cardiaque et maladie de Parkinson, *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1914, No. 14, p. 599.

15. Miloslavich, E.: Ueber Trigemini-vagus Reflex, *Wien. med. Wchnschr.*, 1910, lx, 3051.

16. Loeper, M., and Mougeot, A.: L'absence du réflexe oculo-cardiaque dans le tabes, *Progrès méd.*, 1913, No. 52, p. 675.

17. Loeper, M., and Mougeot, A.: Absence fréquente du réflexe oculo-cardiaque dans le tabes, *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1913, No. 39, p. 942.

18. Lesieur, Ch.: Vernet, M., and Petzetakis: Note sur l'abolition fréquente du réflexe oculo-cardiaque dans le tabes, *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1914, No. 9, 446.

patients the pulse rate is either not slowed at all by ocular pressure or only slightly affected. Lesieur, Vernet and Petzetakis found the reflex to be absent when the Argyll Robertson phenomenon had not yet developed. In syphilis of the nervous system, other than tabes dorsalis, Loeper, Mougeot and Vahram¹⁹ found that the reflex was also absent in thirty of their forty cases.²⁰ None had Argyll Robertson pupils. These patients were generally in the tertiary stage. The reflex, then, is lost both in tabes and in other syphilitic diseases of the nervous system. But there need be no necessary relation between its loss and the presence or absence of the Argyll Robertson pupil. Different results were obtained in this study concerning the condition of the reflex in syphilitic patients who are not tabetic. Further reference will be made to this later.

The condition of the reflex in Basedow's disease has aroused a great deal of interest. Grossmann and Miloslavich,² Milian,⁴ Sainton,²¹ and Lesieur, Vernet and Petzetakis¹⁴ have shown that it is generally exaggerated in this condition. They attribute this to the hypervagotonicity present in these patients.

The condition of the reflex in tachycardias and bradycardias has been studied by Mougeot²² and Loeper and Mougeot.²³ They believe, in general, that in tachycardias and in bradycardias of nervous origin, the reflex is preserved, while in those of myocardial origin, it is lost. They suggest this difference as a method for distinguishing the two. Fabre and Petzetakis²⁴ observed that in the

19. Loeper, M., Mougeot, A., and Vahram: Abolition fréquente du réflexe oculo-cardiaque chez les syphilitiques, *Progrès méd.*, 1914, No. 14, p. 157.

20. The authors give no reason why the cases should be considered syphilis of the nervous system and not merely tertiary syphilis. They conclude, however, that the frequent absence of the oculocardiac reflex is evidence that the nervous system is early involved.

21. Sainton: Le réflexe oculo-cardiaque dans la syndrome de Basedow, *Bull. méd.*, 1913, No. 60, p. 701.

22. Mougeot, A.: Tachycardie paradoxale des hypertendus et réflexe oculo-cardiaque, *Progrès méd.*, 1913, No. 51, p. 663; Le réflexe oculo-cardiaque dans les tachycardies permanentes sans arythmie, *Compt. rend. Soc. de biol.*, 1914, No. 5, p. 205.

23. Loeper, M., and Mougeot, A.: Le réflexe oculo-cardiaque dans le diagnostic de la nature des bradycardies, *Compt. rend. Soc. de biol.*, 1914, No. 3, p. 104.

24. Fabre and Petzetakis: De la bradycardie et automatisme ventriculaire provoqué dans les suites de couches par la compression oculaire, *Réunion Obstétrical et Gynecologique de Lyon*, January 19, 1914, p. 37.

bradycardias occurring during the puerperium, ocular pressure caused auriculoventricular dissociation. Dufour and Legras²⁵ reported the case of a young woman who, having had a miscarriage a few months before, suddenly developed hypo-ovarian symptoms; that is to say, amenorrhea and loss of hair. Later she developed hyperthyroidism, indicated by the presence of exophthalmos and tachycardia. Epileptiform attacks, due, it is said, to cerebrospinal hypertension, supervened. During this time ocular pressure caused no slowing of the heart. Later, when the hair began to return, the reflex reappeared, so that during ocular pressure complete auriculoventricular dissociation took place. No mention was made as to which eye caused the dissociation. The authors do not clearly state whether the reflex was absent before hyperthyroidism developed, during it, or after the symptoms had quieted down. It does not seem likely that the occurrence of hyperthyroidism should make the reflex disappear. Its absence was more probably due to the hypo-secretion of the ovaries; for the reflex reappeared when the ovaries functioned normally.

The influence of drugs on the condition of the reflex has been studied by Petzetakis²⁶ and Mougeot.²⁷ They found that atropin 0.02 mg.²⁸ injected subcutaneously abolished the reflex for a period of one to three hours. In a small portion of cases atropin exaggerated the reflex or excited automatic ventricular beats. These are the cases in which atropin retards rather than accelerates the

25. Dufour and Legras: Réflexe oculo-cardiaque provoquant l'arrêt du coeur, l'automatisme ventriculaire et la dissociation auriculo-ventriculaire, Bull. et mém. Soc. méd. d. hôp. de Paris, 1914, No. 14, p. 686.

26. Petzetakis: L'épreuve de l'atropine, du nitrite d'amyl et de la compression oculaire dans les bradycardies totales, Compt. rend. Soc. de biol., 1913, lxxv, p. 677; De l'automatisme ventriculaire provoqué par la compression oculaire et l'atropine dans les bradycardies totales, Compt. rend. Soc. de biol., 1914, No. 1, p. 15; L'épreuve de la compression oculaire du nitrite d'amyl et de l'atropine dans le diagnostic des bradycardies totales d'origine nerveuse, Presse méd., 1914, No. 17, p. 161; Abolition du réflexe oculo-cardiaque par l'atropine; son exagération par la pilocarpine; sa persistance pendant l'épreuve du nitrite d'amyl, Compt. rend. Soc. de biol., 1914, No. 6, p. 247; Le réflexe oculo-cardiaque chez les sujets normaux non bradycardiques, Bull. et mém. Soc. d. hôp. de Paris, 1914, No. 12, p. 562.

27. Mougeot, A.: Suppression constante par l'atropine de réflexe oculo-cardiaque, Compt. rend. Soc. de biol., 1914, No. 4, p. 162.

28. This seems to be a small dose.

heart rate. Pilocarpin, 0.01 gm. generally exaggerated the reflex. When amyl nitrite was given ocular pressure slowed the accelerated heart, but inhibition began after a longer latent period than occurs normally.

Various studies of the effect of the ocular pressure on the mechanism of the heart itself have been made. Mougeot²⁹ found that the reflex was generally not abolished during alternation of the ventricle. In some cases of uremia and other intoxications in which ventricular alternation was present, the reflex was lost. Its reappearance was taken to indicate amelioration in the underlying condition. It has been suggested that the loss of the reflex depends on an intoxication of the vagus nuclei in the medulla, by means of which the path of the reflex is cut. In ten of seventy-five cases, Petzetakis^{28, 30} obtained transient dissociation of the auricles and ventricles by ocular pressure. The same arrhythmia was also observed by Gallavardin, Dufour and Petzetakis.³¹ No mention was made of any difference between the effects produced by pressure on the right and left eyes. A little later Petzetakis³² was able to conclude from a study of two cases that pressure on the left eye is more apt to cause disturbance in auriculoventricular conduction, while pressure on the right eye has a greater influence on rate. His explanation for the difference in effect between the two is that the right vagus is more sensitive than the left. He found two patients in whom ocular pressure caused partial heart-block and later complete auriculoventricular dissociation. The arrhythmia was prevented from appearing in these cases by the injection of atropin. Petzetakis³³ reported one case in which atropin had the reverse effect. In

29. Mougeot, A.: Le réflexe oculo-cardiaque dans l'alternance ventriculaire, *Compt. rend. Soc. de biol.*, 1914, No. 12, p. 541.

30. Petzetakis: Réflexe oculo-cardiaque et dissociation auriculo-ventriculaire, *Compt. rend. Soc. de biol.*, 1914, No. 10, p. 409; Automatismes ventriculaires intermittents provoqués à l'état normal, *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1914, No. 14, p. 727.

31. Gallavardin, Dufour and Petzetakis: Automatismes ventriculaires intermittents, spontanés ou provoqués par la compression oculaire et l'injection d'atropine dans les bradycardies totales, *Arch. de mal. du coeur*, 1914, vii, 1.

32. Petzetakis: Block auriculo-ventriculaire provoqué par la compression oculaire, *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1914, No. 14, p. 739.

33. Petzetakis: L'épreuve paradoxale de l'atropine. Son action ralentissante sur le rythme cardiaque, *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1914, No. 12, p. 567.

this case the injection *slowed the pulse* and permitted the appearance of the reflex, which had not been present before. In most instances diagnoses of the disorders of the heart mechanism were made by means of polygraphic tracings. This method does not give conclusive information on some points of cardiac arrhythmias, and when the tracings are made from patients disturbed by manipulation, they involve some doubt.

Pressure over the vagus nerves in the human subject has been studied particularly by Robinson and Draper.³⁴ They found that vagal pressure caused slowing of the auricles and ventricles and depressed conductivity in cases having normal hearts. The *Q-B* interval, i. e., the time between the *Q* wave in the electrocardiogram and the footpoint of the brachial pulse, was sometimes diminished. The right vagus had a greater effect on the force and rate of the heart, while the left vagus had a more pronounced effect on the conduction of impulses from auricles to ventricles. The heart was found to respond more quickly to right than to left vagal pressure. Right vagal pressure caused an increase in the action current of the ventricles and a decrease of the current of the auricles, i. e., the height of the *R* waves was increased and the height of the *P* waves was diminished. Left vagal pressure diminished the height of the *R* waves, while the *P* waves were unaffected. In cases of auricular fibrillation, vagal pressure slowed the ventricles without affecting the auricles. Reference will later be made to these results.

Method.

In this investigation, patients were placed in the galvanometer circuit, and electrocardiograms were made to serve as controls. An assistant at the bedside, in continuous telephonic communication with the operator at the galvanometer, exerted pressure on the neck, or on the eye, as the case might be. Enough pressure was exerted on the neck entirely to obliterate the carotid pulse. No accurate measurement of the degree of pressure exerted on the eye was possible. An attempt was made later to determine the amount of pres-

34. Robinson, G. C., and Draper, G.: Action of Vagus Nerve on Human Heart, *Jour. Exper. Med.*, 1911, xiv, 217; Studies with the Electrocardiogram on the Action of the Vagus Nerve on the Human Heart, *Jour. Exper. Med.*, 1912, xv, 14.

sure, in millimeters of mercury, used on the eyes in order to obtain a standard for comparison with other observers. As nearly as could be judged, a similar degree of pressure was applied to the distended air-tight bag of a blood-pressure apparatus. By this method the pressure employed was found to be approximately 30 mm. of mercury. The eyelid was first closed and the operator's thumb applied under the supraorbital ridge, but not directly over the cornea. The pressure on the two eyes was made as nearly equal as possible. Before pressure was made a strip of curve of seven or eight seconds' duration was taken. A signal registered the onset of pressure on the tracings. Pressure continued for about six or seven seconds, the offset being marked by the second signal in the tracing. The exposure was continued about eight or ten seconds longer to include the return of the heart's action to normal. In most cases the second lead was taken (right arm to left leg), but in some instances the first lead (right arm to left arm) was tried, to obtain, if possible, more prominent auricular waves.

Pressure was usually made first on the right vagus, then on the left vagus, then on the right eye and last on the left eye. Two records of each pressure (eight in all) were obtained. Four minutes were permitted to elapse between any two pressures, in order to exclude the summed influence of one on the succeeding. Occasionally, the left vagus was pressed on first and sometimes the left or right eye, to be certain to exclude confusion occasioned by fatigue phenomena. The same patient was in a few instances examined on two different days, and the order of pressure was reversed. Different degrees of pressure were used in the same patient at different times in order to determine whether the amount of slowing varied with the intensity of the pressure. Finally, the symptoms of the patient during pressure were recorded. The signs found included flushing of the face, inhibition of respirations, expressions of pain and the movements of deglutition, etc.

*Protocols.*³⁵

CASE 1.—School boy, aged 9 years. He had a rheumatic history. Pupils react normally.

35. The protocols have been compiled to report the sex, age, occupation, previous diseases having a possible influence on the cardiovascular system, the state of the pupils at the time of examination, and the clinical diagnosis.

Clinical Diagnosis: Chorea, rheumatic fever, chronic valvular disease.

CASE 2.—School boy, 11 years old. Pupils react normally.

Clinical Diagnosis: Chronic endocarditis.

CASE 3.—Clerk, 49 years old. No rheumatic history. Pupils react normally.

Clinical Diagnosis: Rheumatic fever, myocardial insufficiency, auricular fibrillation.

CASE 4.—Printer, 49 years old. No rheumatic history. Pupils equal, oval, and react sluggishly to light. Reaction to accommodation is greater and more rapid.

Clinical Diagnosis: Tabes dorsalis.

CASE 5.—Business man, 38 years old. Pupils are equal and react sluggishly to light. Both react well in accommodation.

Clinical Diagnosis: Syphilitic aortitis and syphilis of the spinal cord (syphilitic myelitis).

CASE 6.—Male, 49 years old. Instructor in physical culture. Pupils are equal, small, do not react to light, prompt reaction in accommodation.

Clinical Diagnosis: Tabes dorsalis.

CASE 7.—Coachman, 45 years old. Pupils unequal, right is 2 mm., left is 5.5 mm. in diameter. Neither reacts to light and both have sluggish reaction in accommodation.

Clinical Diagnosis: Syphilis of the brain and spinal cord (probably early paresis or taboparesis).

CASE 8.—Salesman, 46 years old. Pupils unequal, right slightly larger than left. Both irregular. React slightly but sharply to light and normally in accommodation.

Clinical Diagnosis: Tabes dorsalis.

CASE 9.—Blacksmith, 58 years old. Rheumatic fever four years ago. Hard chancre thirty years ago. Pupils react normally.

Clinical Diagnosis: Acute rheumatic fever, acute pericarditis, cardiac arrhythmia, transitory auricular fibrillation, serofibrinous pleurisy and latent syphilis.

CASE 10. Laborer, 31 years old. Rheumatic fever seven years ago, and two attacks since. Pupils react normally.

Clinical Diagnosis: Chronic valvular disease.

CASE 11.—School girl, 11 years old. Had one attack of rheumatic fever. Pupils react normally.

Clinical Diagnosis: Chronic mitral regurgitation. Chronic cardiac hypertrophy and dilatation.

CASE 12.—School girl, 9 years old. Rheumatic fever five years ago. Pupils react normally.

Clinical Diagnosis: Acute rheumatic fever, chronic valvular disease, mitral regurgitation, aortic regurgitation and aortic stenosis.

CASE 13.—School boy, 12 years old. Pupils react normally.

Clinical Diagnosis: Chronic valvular disease, mitral regurgitation and stenosis, chronic cardiac hypertrophy and dilatation.

CASE 14.³⁶—Tailor, 30 years old. Right pupil larger than left. Reaction to light and accommodation is active in both eyes.

Clinical Diagnosis: Secondary syphilis, syphilitic periostitis of the frontal bone, periostitis of ulna; multiple gonorrheal, periurethral abscesses.

CASE 15.—School boy, 14 years old. Rheumatic fever. Pupils react normally.

Clinical Diagnosis: Acute rheumatic fever and acute endocarditis.

CASE 16.—School girl, 11 years old. Chorea and rheumatic fever. Pupils react normally.

Clinical Diagnosis: Lobar pneumonia.

CASE 17.—Male, 9 years old. School boy. No history of rheumatic fever. Pupils react normally.

Clinical Diagnosis: Lobar pneumonia and empyema.

CASE 18.—Cloakmaker, 40 years old. Pupils slightly dilated and unequal. Left is 4.5 mm., right is 4 mm. in diameter. Both irregular and react in accommodation but not to light.

Clinical Diagnosis: Tabes dorsalis.

CASE 19.—Bookkeeper, 63 years old. No history of rheumatic fever. Pupils equal and react in accommodation but not to light.

Clinical Diagnosis: Tabes dorsalis.

CASE 20.—Electrician, 32 years old. Pupils unequal. Right is twice the size of the left. Both react slightly to light and well in accommodation.

Clinical Diagnosis: Tabes dorsalis.

CASE 21.—Business man, 26 years old. Rheumatic history eight years ago. Pupils slightly irregular in outline but react normally.

Clinical Diagnosis: Secondary syphilis. Syphilis of cerebrospinal meninges.

CASE 22.—Girl, 17 years old. Machine operator. Pupils react normally.

Clinical Diagnosis: Diabetes mellitus.

CASE 23.—Man, 53 years old. Actor. Pupils are unequal; right 3 mm., left 2.25 mm. in diameter. Neither reacts to light, but both react in accommodation.

Clinical Diagnosis: Tabes dorsalis.

CASE 24.—Reporter, 34 years old. Pupils are equal, irregular in outline, react normally to light and in accommodation.

Clinical Diagnosis: Secondary syphilis, syphilis of the cerebrospinal meninges, syphilis of the auditory and facial nerves.

CASE 25.—Tailor, 53 years old. Pupils equal, slightly irregular, do not react to light; both react slightly in accommodation.

Clinical Diagnosis: Tabes dorsalis.

CASE 26.—Porter, 35 years old. Pupils react normally.

Clinical Diagnosis: Lobar pneumonia and lung abscesses.

CASE 27.—Boy, 18 years old. Factory hand. Pupils react normally.

Clinical Diagnosis: Lobar pneumonia.

CASE 28.—Salesman, 24 years old. Pupils react normally.

Clinical Diagnosis: Lobar pneumonia.

36. Case 14 has been omitted because he began to show signs of early tabes, although the oculocardiac reflex was present.

CASE 29.—Diamond cutter, 60 years old. Had syphilis thirty-eight years ago. Pupils are somewhat dilated. Left does not react to light as completely and quickly as right. Both react well in accommodation.

Clinical Diagnosis: Transient heart-block. Syphilis of the heart.

CASE 30.—Farm hand, 26 years old. Pupils react normally.

Clinical Diagnosis: Acute rheumatic fever, acute myocarditis, lobar pneumonia.

General Results and Observations.

The material for this study consisted of eight cases of *tabes dorsalis*, one case of *taboparesis*, five cases of syphilis which were non-tabetic, nine cases of chronic endocarditis (having a normal rhythm), one case of chronic endocarditis with auricular fibrillation, five cases of lobar pneumonia, and one case of diabetes mellitus. The five non-tabetic syphilitics included two cases of syphilis of the cerebro-spinal meninges, one of syphilitic myelitis, one of syphilis of the heart, and one patient who has been observed during the secondary stage and who now suggests signs of early *tabes*.

The results of ocular and vagus pressure from four (Cases 4, 18, 20 and 25) of the eight cases of *tabes dorsalis* are shown in detail in figures 1a-d. Figure 1a gives the results of right vagus pressure, 1b of right ocular pressure, 1c of left vagus pressure and 1d of left ocular pressure. In one instance (Figure 1c, Tracing 157) there is marked slowing on vagus pressure. The degree of inhibition of the heart produced by vagus pressure was greater in this case than in any of the other cases (tabetic or non-tabetic).³⁷ This is a significant point, because although the oculocardiac reflex will be shown to be generally absent in tabetics and present in non-tabetics, the greatest degree of slowing by direct vagal pressure was obtained in a tabetic. The centrifugal path of the reflex was intact. One (Case 6) of the tabetics, whose pupils were rigid and did not react to light, developed a moderate amount of slowing as a result both of right and left ocular pressure. In some of the other tabetics, where the oculocardiac reflex was absent, the pupils reacted slightly to light. All the other tabetics gave no slowing on ocular pressure. In eight cases of *tabes dorsalis*, then, one showed a moderate oculocardiac reflex whereas it was absent in the other seven. There is, also, no

37. The term non-tabetic is used throughout to include all the cases in the series except those of *tabes dorsalis*.

exact parallelism between the Argyll Robertson reaction and the oculocardiac reflex. There was no difference in the effect produced by pressure on the two eyes. The only change noticed in the electrocardiograms was a slight increase in the prominence of the *P*-waves in two instances on right vagal pressure. There was no marked change in the conduction time or in the form of the ventricular complex.

In the second group of cases are included the four *syphilitics*³⁸ who were *not tabetic*. (Figs. 2a-d.) In three of the four cases, the oculocardiac reflex was preserved. The results obtained here differ, therefore, from those obtained by Loeper, Mougeot and Vahram.¹⁹ They found that in 75 per cent. of their cases of syphilis the oculocardiac reflex was *absent*. The reflex was *present* in 75 per cent. of the cases in this study. In two (Cases 21 and 24) of the four cases (Fig. 2b, Tracings 166 and 187, and Fig. 2d, Tracings 167 and 183) there was complete inhibition of the heart for periods ranging from 3.31 seconds to 10.92 seconds (Fig. 5). In a third case (No. 29) there was a moderate amount of slowing, that is to say, a slight reaction. The fourth (Case 5) did not react at all to ocular pressure. This case was one of syphilis of the spinal cord and syphilitic aortitis. He showed an Argyll Robertson pupil, so that it is not at all unlikely that he had early tabes. The slowing in these cases was more pronounced during pressure on the right ocular bulb than on the left. There were no changes in the electrocardiograms. In Case 21 an escaped ventricular beat occurred 0.79 second after the preceding pause of 3.67 seconds. Another long pause of 2.40 seconds followed. The escaped ventricular beat was of normal form, probably supraventricular in origin. In this case all the waves were greatly diminished in height during ocular pressure, so that it was difficult to differentiate a ventricular contraction from the oscillations that are sometimes observed in electrocardiograms during stimulation of the vagus nerves. Case 24 was very sensitive to ocular pressure. The pacemaker was entirely inhibited by right ocular pressure for 10.92 seconds (Fig. 5). Auriculoventricular conduction was lengthened considerably during pressure on

38. Case 14, originally included here, has been omitted because he began to show signs which may be those of early tabes, although the oculocardiac reflex was present.

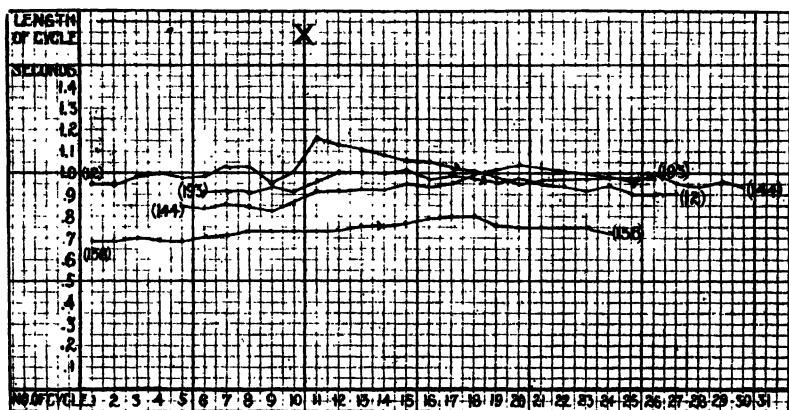


Figure 1a.

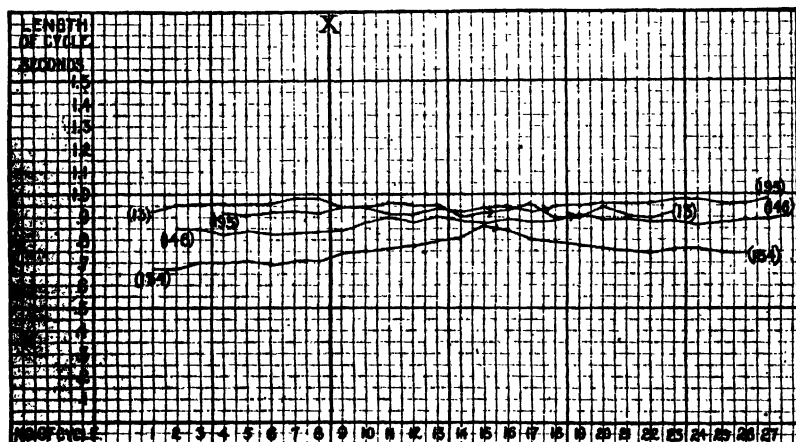


Figure 1b.

Figures 1, 2, 3 and 4 detail the duration (on the ordinate) of every cardiac cycle (on the abscissa) calculated from electrocardiograms taken during a given digital (vagal or ocular) pressure. X indicates the onset of pressure; the arrow (→) the time of the offset. The figures marked *a* show the results of right

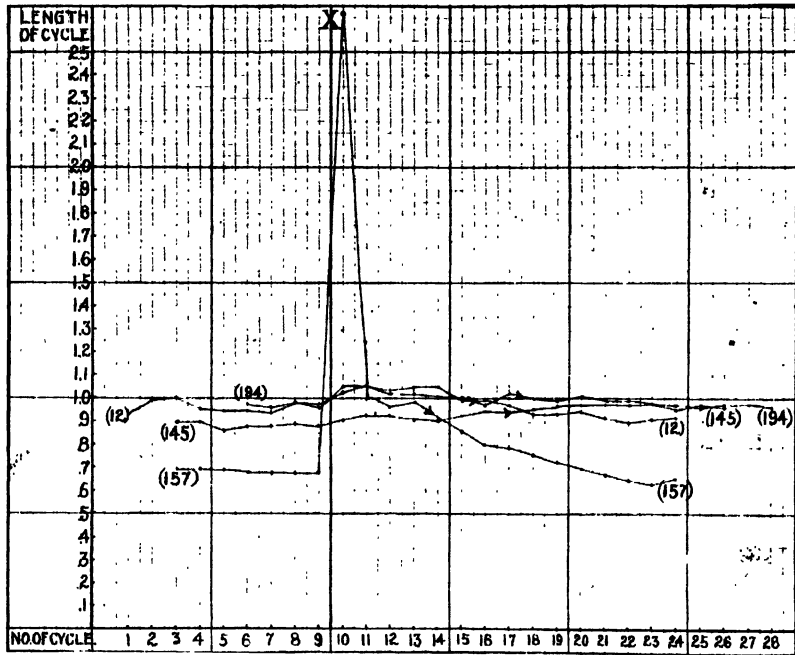


Figure 1c.

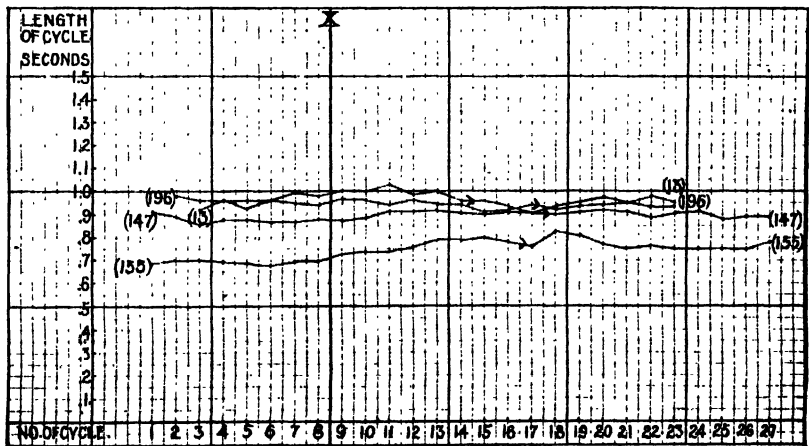


Figure 1d.

vagus pressure; *b*, right ocular pressure; *c*, left vagus pressure; *d*, left ocular pressure. The numbers at the beginnings and ends of the curves indicate, in the accompanying table, the patients to whom the records refer.

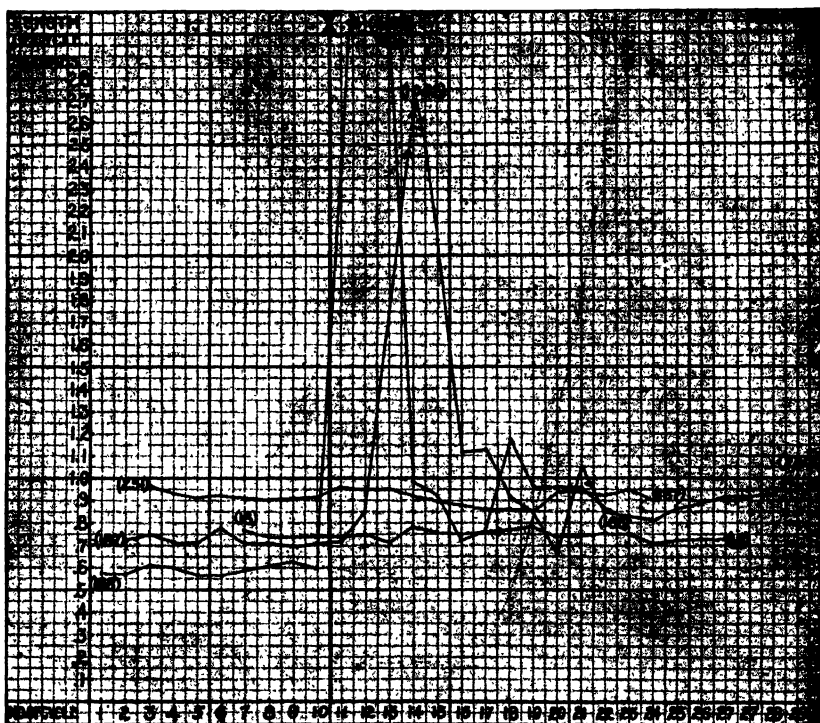
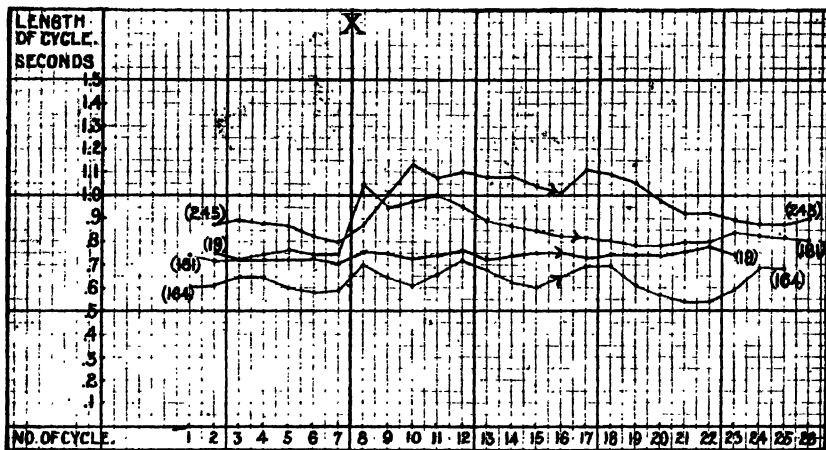


Figure 2b.

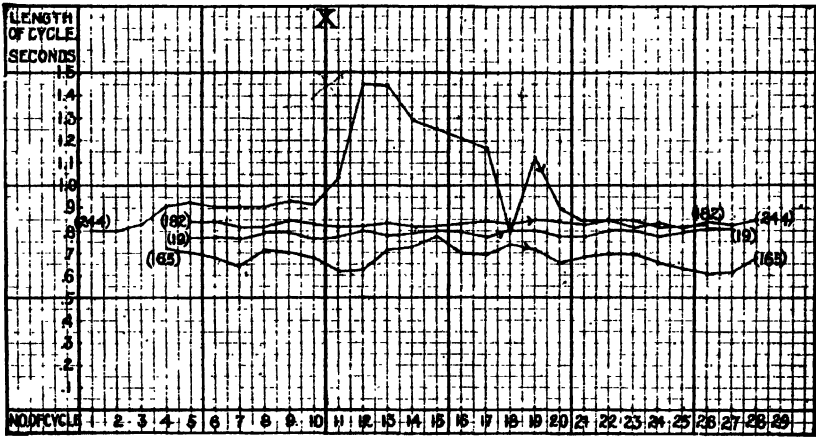


Figure 2c.

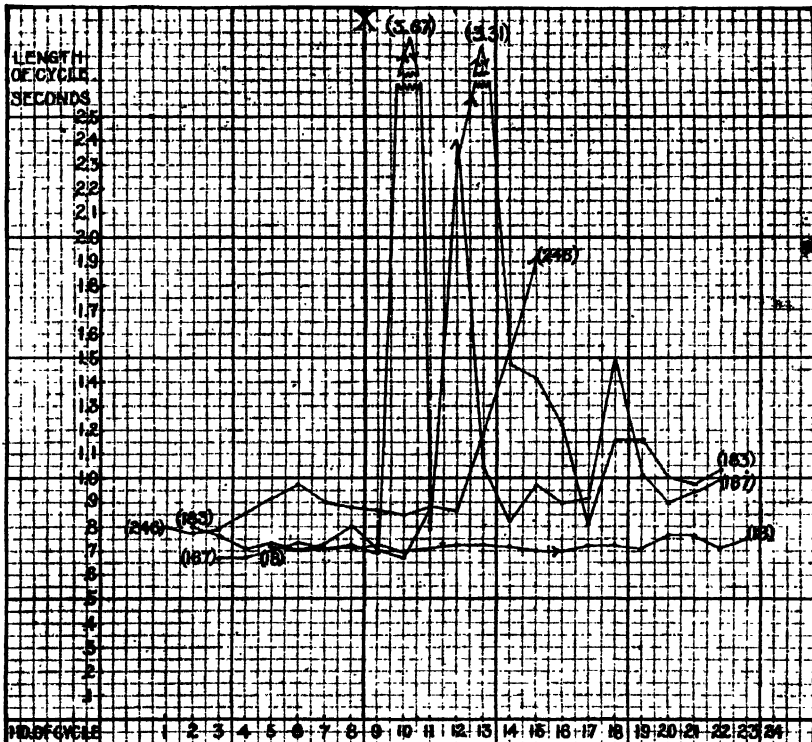


Figure 2d.

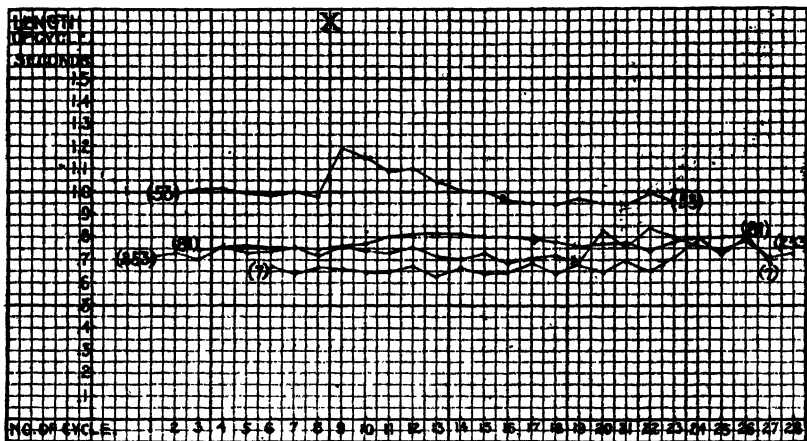


Figure 3a.

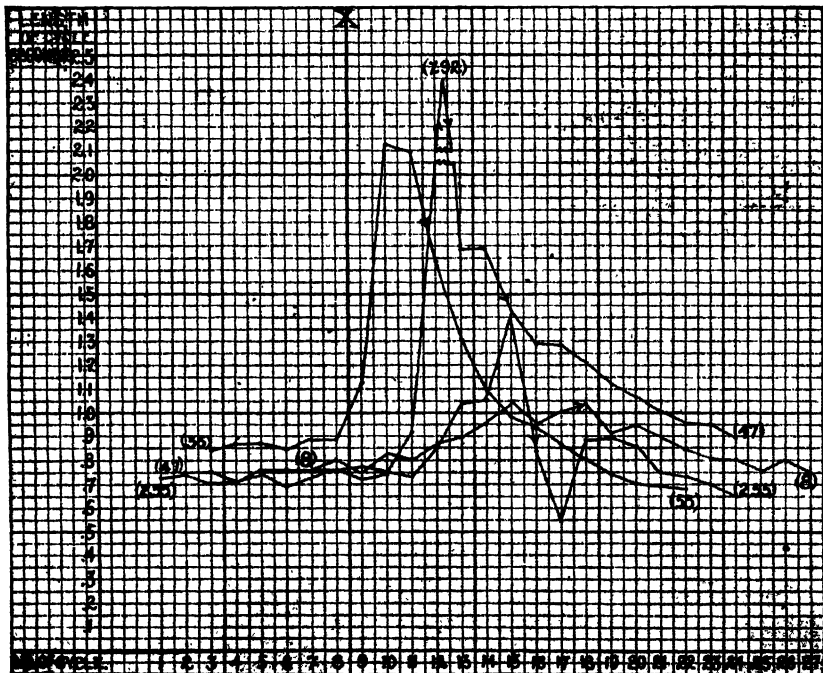


Figure 3b.

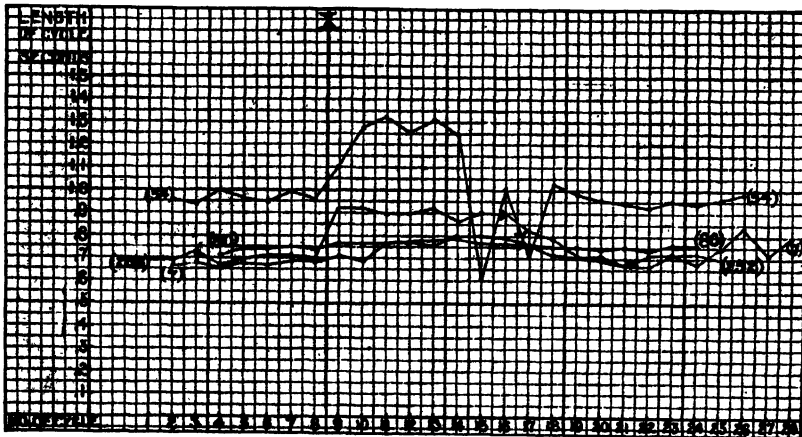


Figure 3c.

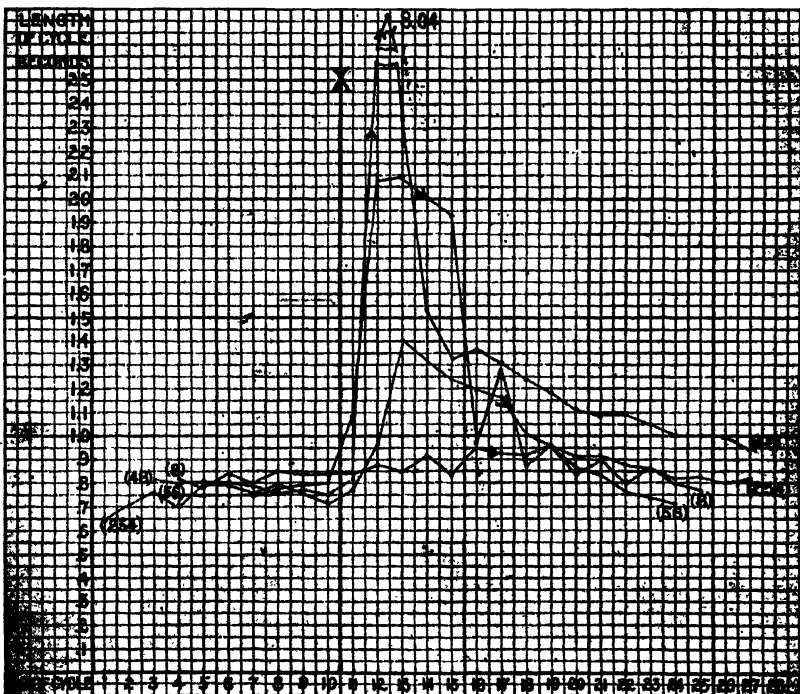


Figure 3d.

the left eye. The effect on conduction, however, was not limited to pressure on the left eyeball, for the *P-R* time following the pause of 10.92 seconds was 0.24 second.

The results of vagal and ocular pressure were also studied in five cases of *pneumonia* (Cases 17, 26, 27, 28, 30). The curves plotted from electrocardiograms taken during vagal pressures rose very

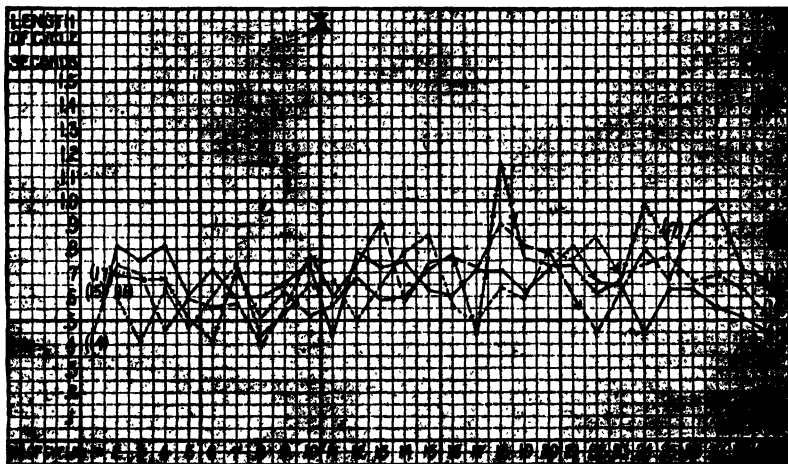


Figure 4a.

Figure 4.—a and b give the results of pressure in a case (No. 3) of auricular fibrillation, before (a) and during (b) the administration of digitalis; a 14 + b 201 ——— right vagus; a 15 + b 208 ——— right eye; a 16 + b 202 ——— left vagus; a 17 + b 209 x—x—x left eye.

slightly, that is to say, pressure did not slow the heart very much. The curves of ocular pressure, on the other hand, rose abruptly after a latent period of from one to three beats. This demonstrates strikingly how much more effective ocular pressure is in slowing the heart than direct vagal pressure. Another point to be observed is that the pulse rate did not drop back to normal after pressure was released, but in fact was still quite slow after ten seconds, and in some instances after thirty seconds. This occurrence emphasizes the importance of waiting between succeeding pressures on the same patient, to guard against either fatigue phenomena or summation of stimuli. One of the pneumonia patients, Case 17, showed

only slight slowing. The reason for this is, probably, that not enough pressure was exerted.

Three (Cases 17, 28 and 30) of the five pneumonia cases showed no changes in the form or sequence of the curves, except the slowing. In the other two, several interesting changes were observed. In Case 26 left ocular pressure caused considerable delay in conduction

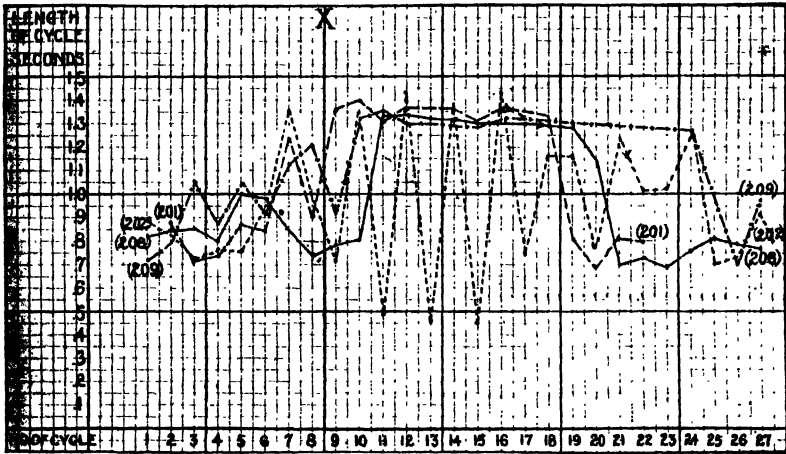


Figure 4b.

time. In places auricular waves cannot be distinguished at all, and yet the ventricular complexes are of normal contour. The auricular representation is iso-electric.

The last of the pneumonia cases (No. 27) presented varied changes during ocular pressure. Figure 8 shows three inverted *P*-waves, one of which was blocked. (Inverted *P*-waves were seen in one other instance during left ocular pressure.) The same tracing showed a conduction time of 0.28 second. On five different occasions left ocular pressure caused delayed conduction, the *P*-*R* interval rising to 0.24 and 0.32 second. In none of the four times when the right eye was pressed in this case was there any delay in conduction. Figure 6 shows partial heart-block as a result of left ocular pressure. Finally, in one instance, pressure on the left bulb caused an increase in the height of the *P*-waves, an occurrence observed also by Robinson and Draper³⁴ as a result of direct pressure

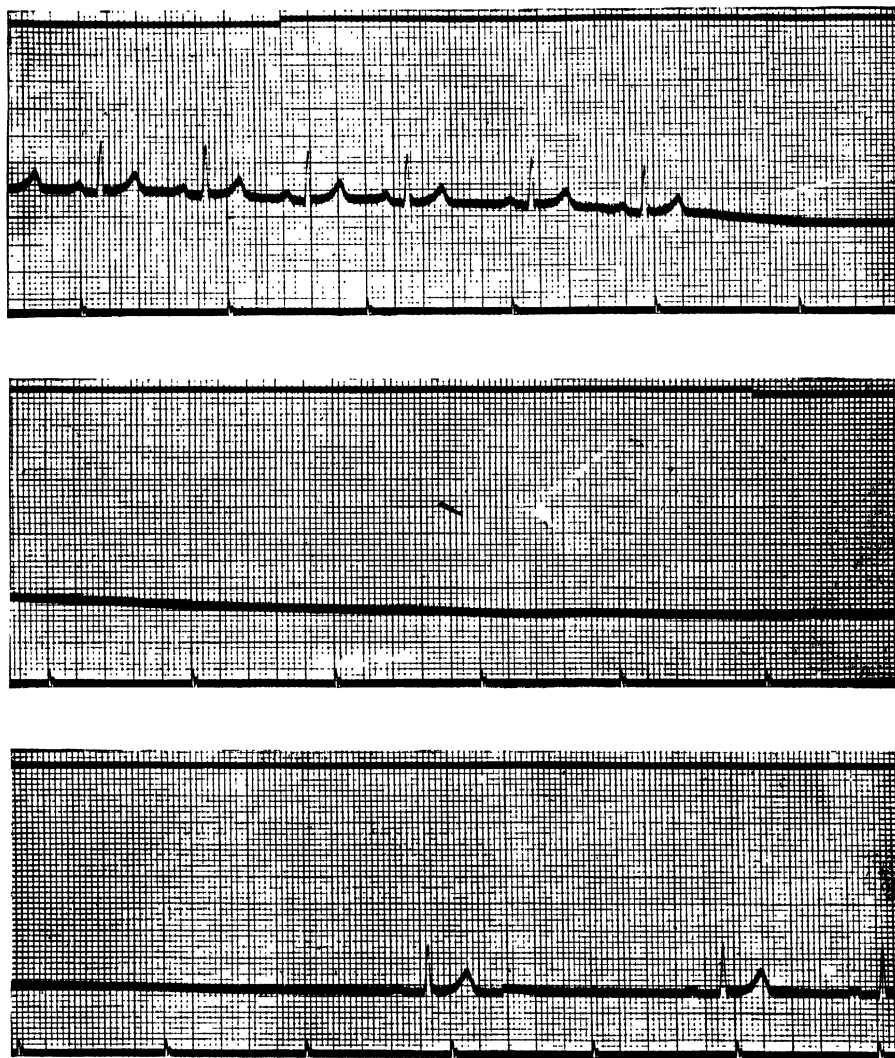


Fig. 5.—Case 24. Right ocular pressure. An electrocardiogram showing total inhibition of all chambers of heart for 10.92 seconds. The *P-R* time of the beat after the long pause is lengthened to 0.24 second. The slowing continued for twenty-four seconds after the release of pressure. In all curves, divisions on the abscissa equal 0.04 second. Above is a signal indicating the duration of digital pressure. Below a time signal marking one-second intervals.

on the left vagus nerve. In this case, then, right ocular pressure merely slowed the heart and had no effect on conduction. Pressure on the left eye, however, caused ectopic auricular contractions, delayed conduction, partial heart-block, and an increase in the height of the *P*-waves. These different effects appeared in the same individual under similar circumstances. It seems reasonable to believe that the duration and the force of the pressure, which must have varied, were important factors in determining the degree of inhibition that took place. Just as increasing strengths of faradic currents applied to exposed nerves in experiments on animals are accompanied by increasing effects, so may increasing pressures produce increasing effects on the rate and on conduction. Mild pressures may slow the rate and perhaps slightly affect conduction; slightly greater pressures may block auricular beats, and great pressures may stop the heart. Under certain circumstances, possibly during removal of the pressure, complete auriculoventricular dissociation might occur. It is therefore important to standardize the degree and duration of pressure. In addition, the resistance offered by the eye is probably a factor involved in eliciting the reflex.

Nine cases of *chronic valvular disease* (Patients 1, 2, 9, 10, 11, 12, 13, 15, 16), all of whom had hearts actuated by a normal rhythm, were studied. Figures 3a-d show the results in four of them (1, 2, 9 and 10). Only one (Case 10) of the nine cases had a moderate reaction to *vagal pressure*, while another (Case 1) showed a slight effect. All the others were little, if at all, affected by either right or left vagal pressure. On *ocular pressure*, eight (Nos. 1, 9, 10, 11, 12, 13, 15 and 16) reacted strikingly, and the other one (No. 2) moderately. The patient (No. 2) who reacted with moderate slowing, was not pressed very vigorously; he was one of the first patients studied. The first (No. 1) patient studied showed no slowing whatever on moderate ocular pressure, but on greater pressure he had a good reaction. In this case *P*-waves diminished in height during right ocular pressure, and during left ocular pressure *P*-waves were blocked. Left ocular pressure caused delayed conduction (*P-R* interval 0.44 second in one instance). Right vagal pressure caused a slight delay in conduction on one occasion. There was no noticeable difference on the heart rate between the effects of right and left ocular pressure in these cases.

The results observed in one patient (Case 3) whose heart showed the rhythm of *auricular fibrillation* are included in the study. Observations were made before and during the administration of digitalis. Figure 4a shows the results of ocular and vagal pressures before digitalis was given. There was no evidence of slowing during pressure. Figure 4b, which gives the results after a course of digitalis therapy, shows a distinct slowing on pressure. There was no difference in effect between the right and left sides or between ocular and vagal pressure either before or during digitalis treatment. All other non-tabetic cases showed a distinct difference in reaction between ocular and vagal pressure. Many of them manifested differences in the effect of pressure on the right and left eyes. But here the result was the same whether ocular or vagal pressure was

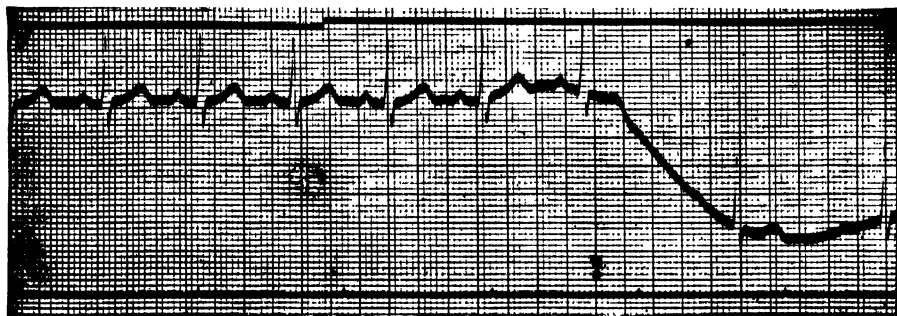


Figure 6.

Fig 6.—Case 27. Left ocular pressure. Moderate slowing of the heart is shown. There is a decrease in the size of the *P* wave. Conduction time is delayed and is gradually increased until an auricular beat is blocked. The *P-R* interval following the blocked beat is short, 0.15 second.

exerted or whether it was the left or right side that was used. The up and down swings in Figure 4b, Curve 202, are due to three premature ectopic ventricular beats alternating with the normal ventricular contractions. There is a striking tendency for all normal heart cycles in this chart to be of the same length during pressure. Digitalis, it seems, changed the condition of the oculocardiac reflex. Wenckebach³⁹ and v. Hoesslin⁴⁰ have similarly shown that digitalis produces a heightening of the efficiency of vagus pressure.

39. Wenckebach, K. F.: *Die Unregelmässige Herzthätigkeit und ihre klinische Bedeutung*, W. Engelmann, Leipzig and Berlin, 1914, p. 172.

40. Von Hoesslin, H.: *Beobachtungen über den Einfluss des Vagus auf das menschliche Herz*, *Deutsch. Arch. f. klin. Med.*, 1914, cxiii, 537.

One case of diabetes mellitus was studied (Case 22). Tracings were taken two different times at an interval of fifteen days. At neither time was there more than the slightest slowing either during vagal or ocular pressure. There has been no report in the literature of the condition of the oculocardiac reflex in this disease. A study of more cases is required to decide whether the condition of the reflex found in this case is typical.⁴¹

The sensations experienced and the objective signs displayed during pressure have been studied to determine if differences between tabetics and non-tabetics were present, and to see if they were constant. Of the thirty patients studied, six had unusual sensations of one kind or another (Table 1). The others felt nothing but the pressure and the pain, when there was any. An examination of

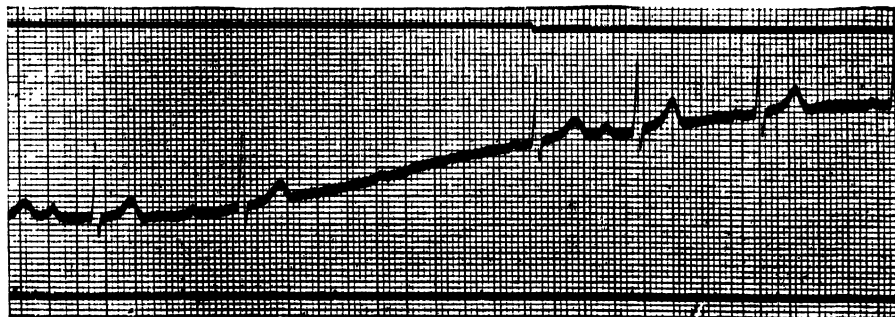


Figure 6—Continued.

Table 1 shows that *vagus pressure* had little effect on either tabetics or non-tabetics. Occasionally a patient complained of slight pain; very rarely there was flushing or inhibition of respiration; on a few occasions the patient swallowed during or immediately after pressure. Deglutition might very well have resulted from the discomfort of the pressure on the neck. *Ocular pressure*, on the other hand, produced a marked difference in the two groups of cases. In the tabetics, three⁴² had a moderate amount of pain (marked +),

41. Since these observations were made, three cases of diabetes in the medical wards of the Peter Bent Brigham Hospital were investigated. In one case the oculocardiac reflex was absent on two different occasions. In the other two there was a moderate slowing on right ocular pressure, but none on the left.

42. I have included under tabetics Case 7, which is probably a case of early taboparesis.

TABLE I.
Sensations Elicited by Pressure in Tabetics and Non-Tabetics.
Tabetics.

Case	Vagus				Eye				Subjective Symptoms
	Pain	Flushing	Inhibition of Respiration	Deglutition	Pain	Flushing	Inhibition of Respiration	Deglutition	
4	-	+	-	+	+	sl.	-	-	Felt slight pain with ocular pressure, especially right.
6	-	-	-	-	-	-	-	-	Felt nothing but the pressure. No pain.
7	-	-	-	-	+	-	-	-	Slight pain in eyes. Pins and needles sensation in right eye.
8	-	-	-	-	-	-	-	-	Had no other feeling than that of pressure.
18	-	-	+	-	-	sl.	-	-	Merely felt pressure.
19	-	-	-	-	sl.	sl.	+	-	Merely felt pressure.
20	-	+	-	-	+	sl.	-	-	Pressure on left vagus made him feel faint.
23	-	-	-	-	v. sl.	-	-	-	Felt nothing except slight pain in eyes.
25	-	-	-	-	sl.	sl.	-	-	Felt very slight pain in the eyes.

Non-Tabetics.

1	-	-	-	-	+	+	-	+	Pain in the eyes.
2	..	..	..	..	..	..	..	..	No record obtained.
3	-	sl.	-	-	++	+	-	-	Felt faint, choking sensation, cramp in belly when eyes were pressed.
5	-	-	-	-	-	-	-	-	Felt nothing peculiar. (See protocol.) (May be tabetic.)
9	-	+	-	-	++	+	+	-	Felt nothing but pain from pressure on eyes.
10	-	-	-	-	++	+	+	-	Felt nothing but pain from pressure on eyes.
11	-	-	+	-	++	-	+	-	Felt nothing but pain from pressure on eyes.
12	-	-	-	-	++	+	+	-	Felt nothing but pain from pressure on eyes.
13	+	+	+	-	++	+	+	-	Felt nothing but pain from pressure on eyes.
14	-	-	-	+	++	+	+	-	Felt nothing but pain from pressure on eyes.
15	-	-	-	-	++	+	+	-	Felt nothing but pain from pressure on eyes.
16	-	-	-	-	++	+	+	-	Felt nothing but pain from pressure on eyes.
17	+	-	-	-	++	+	+	-	Felt nothing but pain from pressure on eyes.
21	rt.	rt.	-	-	++	+	+	-	Felt nothing but pain from pressure on eyes.
22	-	sl.	-	-	++	+	+	-	Felt nothing but pain from pressure on eyes.
24	-	-	-	-	++	+	+	-	Felt slow electric current going through body when eyes were pressed. It caused him much pain.

TABLE 1.—*Concluded.*

Case	Vagus				Eye				Subjective Symptoms
	Pain	Flushing	Inhibition of Respiration	Deglutition	Pain	Flushing	Inhibition of Respiration	Deglutition	
26	—	—	—	—	++	+	+	—	Left eye pained more than right.
27	—	—	—	—	++	+	+	—	Left eye pained more than right.
28	—	—	—	—	++	+	+	—	Felt nausea when right eye was pressed, in addition to pain.
29	—	—	—	—	++	+	—	—	Merely felt pain.
30	—	—	—	—	++	+	—	—	When right eye was pressed right leg felt dead and it lifted itself up from bed.

three had a slight or very slight pain (marked v. sl.) and three had no pain whatever (marked —). In five cases there was slight flushing and in four none at all. Respiration was checked during pressure on only two occasions and at no time was there any impulse toward swallowing. In contrast to this are the results in non-tabetics. The pain from ocular pressure was quite severe in all but two cases. One of these was a case of syphilitic myelitis (there is evidence that he is developing tabes), and the second was subjected to much lighter pressure than any of the others. There was flushing of the face in all but two cases. Respiration was checked in fifteen of the twenty cases. It is evident that pain, flushing of the face and respiratory inhibition generally occur during ocular pressure in non-tabetics, and to a much less extent in tabetics.

DISCUSSION AND SUMMARY.

The oculocardiac reflex (inhibition of the heart produced by ocular pressure) is a normal but variable reflex. The statement that a heart is abnormal if its rate is slowed or accelerated by more than ten beats a minute, made by Loeper and Mougeot⁵ is not substantiated by the observations made in this study. Some individuals normally have a more active oculocardiac reflex than others, just as some have more active tendon and superficial reflexes. In fact, when the reflex is considered absent there is often a slight inhibition of the heart, as is shown by a tendency for the curves in Figures 1b and 1d to rise during pressure. It seems highly probable that different effects may be produced in the same individual by changing

the duration and the degree of ocular pressure. This is shown in several cases described in this investigation. The comparison which was instituted made it clear that inhibition of the heart could much more easily be obtained by ocular than by direct vagal pressure. A difference as striking as that which has been observed between the two can only be obtained if the eyes are pressed quite firmly, causing the patient slight distress. Ocular pressure brings out disturbances in the heart mechanism which were not obtained by direct vagal pressure.

The reflex was found to be generally absent in *tabes dorsalis*. The cause for this probably is a lesion in the brain itself. Although in all the cases of *tabes* the pupils were inactive to light or reacted sluggishly, there does not seem to be any constant relation between the occurrence of the Argyll Robertson phenomenon and the loss of the oculocardiac reflex. In fact, the only tabetic who had a moderate oculocardiac reflex, had no pupillary reaction to light whatever. These findings in tabetics are in accord with the results obtained by previous investigators. Tabetics do not complain of pain on ocular pressure to the same extent as non-tabetics, nor do flushing of the face and periods of apnea during pressure occur to as great a degree as in non-tabetics. It is not impossible that these reflex phenomena result from the sensation of pain. Of the five cases of syphilis (Cases 5, 14, 21, 24 and 29), if those patients are excluded who might possibly be developing *tabes*, namely, Cases 5 and 14, then three, or 100 per cent., had a normal oculocardiac reflex; and if the other two are included, all but one, Case 5, or 80 per cent., reacted normally. These results differ decidedly from those of Loeper, Mougeot and Vahram,¹⁹ who found the reflex absent in 75 per cent. of their cases. One case of diabetes mellitus and one case of auricular fibrillation were found to have no oculocardiac reflex. No explanation for its absence can be given in the case of the diabetic. The patient with auricular fibrillation reacted after he was given digitalis. Of all the other cases in this series (including nine cases of chronic valvular disease, five of pneumonia, and five of syphilis) only one (Case 5) did not have an oculocardiac reflex, and he had signs of early *tabes*.

In tabetics there was no difference between the effects of pressure

on the two eyes. In non-tabetics there was a distinct difference. The right reflex had a slightly greater effect on auricular contraction.⁴³ The different grades of inhibition caused by right ocular pressure were, first, a diminution in the height of the *P*-waves with only moderate slowing; second, disappearance of the *P*-waves and slowing of the rate of the ventricular contractions; third, complete inhibition of all the chambers of the heart. Left ocular pressure, on the other hand, may cause similar phenomena, but does not do so as often as right. The *P*-waves frequently could not be identified, but after pressure was released they gradually reappeared (Fig. 7). The ventricular representative, *R*, continued to have a normal form even though the ventricular rate was reduced. It is impossible in these cases to say whether the normal pacemaker was completely inhibited or whether the origin of the impulse for ventricular contractions resided in the junctional tissue. In most instances the pace-making function probably continued to be at the sinus node. In any case the impulse was supraventricular in origin, for the form of the ventricular complexes remained normal. The changes in the mechanism of the heart beat caused particularly by left ocular pressure were delayed conduction, partial heart-block and inversion of the *P*-waves. Complete inhibition of all the chambers of the heart also occurred. Ectopic ventricular beats occurred in two cases during left ocular pressure. Accurate measurements of the heights of the *P*- and *R*-waves before and during ocular or vagal pressure were not made. *P*-waves diminished in height in many instances during both left and right ocular pressure. One case showed a decrease in the height of the *P*-waves even before slowing of the heart began. Occasionally the height of the *P*-wave was increased by left ocular pressure.

Differences between right and left oculocardiac reflexes, such as were obtained by stimulation of the left and right vagus, were not as definite and striking as those obtained in experiments by Cohn.⁴⁴

43. The greater slowing seen in the charts of pressures on the left side is only apparent and not real; it depends on the blocking of auricular beats which were slowed less by left than by right sided pressures. Rates, however, are counted from ventricular contractions.

44. Cohn, A. E.: On the Differences in the Effects of Stimulation of the Two Vagus Nerves on Rate and Conduction of the Dog's Heart, Jour. Exper. Med., 1912, xvi, 732.

TABLE 2.

Patients Referred to in the Curves.

Patient 4	Patient 18	Patient 20	Patient 25	Patient 5	Patient 21	Patient 24	Patient 29	Patient 2	Patient 10	Patient 9	Patient 1
Fig. 1a 12	144	156	193	Fig. 2a 19	164	181	245	Fig. 3a 7	53	81	253
b 13	146	154	195	b 18	166	187	251	b 8	55	47	255
c 12	145	157	194	c 19	165	182	244	c 7	54	80	252
d 13	147	155	196	d 18	167	183	246	d 8	56	48	254

The fact that these differences were not so clearly demonstrated in the oculocardiac reflex must be due to the more complicated pathway pursued by the impulses through the brain. According to the experiments referred to, the right vagus has a greater influence on the

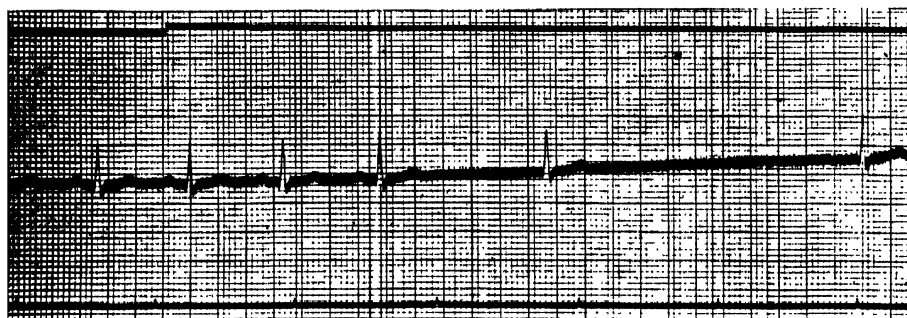


Figure 7.

Fig. 7.—Case 26. Right ocular pressure. Total inhibition of the heart for periods of 2.27 seconds to 3.65 seconds is shown. *P*-waves are entirely absent; the ventricular complexes are of normal form. Beat V is an escaped ventricular contraction of normal form.

pacemaker and the left a greater influence on auriculoventricular conduction. The difference between pressure on the two eyeballs compares satisfactorily, however, with that found by Robinson and Draper³⁴ in their vagus experiments.

CONCLUSIONS.

1. Ocular pressure affords a simple and safe method of obtaining reflex vagus inhibition of the heart.
2. Inhibition of the heart by the oculocardiac reflex is much

more profound and more frequently obtained than by pressure over the vagus nerves.

3. The oculocardiac reflex is generally absent in tabes dorsalis, present in pneumonia, syphilis (non-tabetic) and chronic valvular disease.

4. The reflex was absent in one case of diabetes mellitus and also in one case of auricular fibrillation before digitalis treatment. It was present after digitalis was given.

5. Right ocular pressure has a slightly greater effect on the rate of the heart than left. It may stop the heart for a long period of time, relatively speaking, the *P*-waves are sometimes diminished in

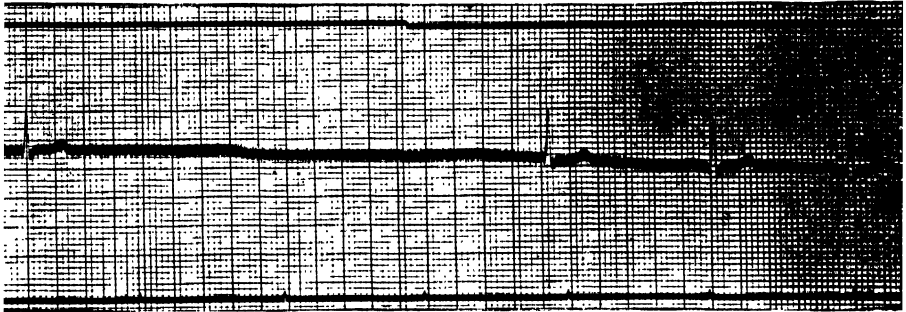


Figure 7—Continued.

size and may become iso-electric. Occasionally the auriculoventricular interval is lengthened.

6. Left ocular pressure has a much greater effect on the conduction mechanism of the heart than right. It may lengthen auriculoventricular conduction, cause partial heart-block and result, possibly, in automatic ventricular rhythm. On two occasions inverted *P*-waves were seen. The height of the *R*-waves is sometimes increased, at other times diminished. Ectopic ventricular beats were twice observed. The *P*-waves are often diminished in size, but occasionally are increased. Escaped ventricular beats were seen both during right and left ocular pressure.

7. Pain, flushing of the face, and apnea during ocular pressure, are much less pronounced in tabetics than in non-tabetics.

8. The effects on the rate and on the rhythm of the heart produced by ocular pressures are not constant, differing in different individuals and in the same individual from time to time. The dura-

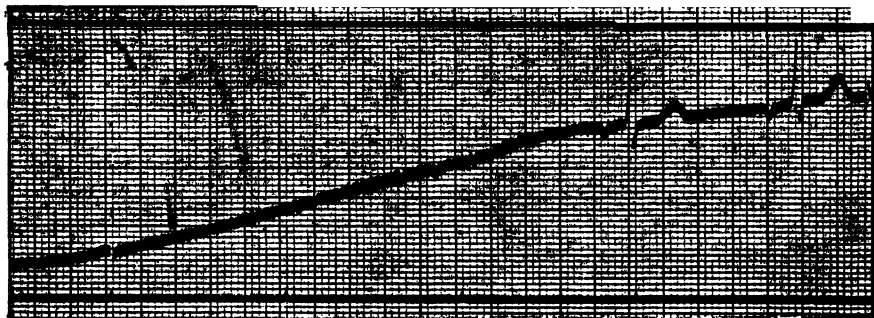


Fig. 8.—Case 27. Left ocular pressure. Inhibition of the ventricle is shown. Three inverted *P*-waves occurred one of which was blocked; in the other two the *P-R* time is short. After release from pressure the *P-R* time was 0.32 second.

tion and the degree of pressure play an important part in the degree of inhibition.

I am greatly indebted to Dr. A. E. Cohn for his help and guidance throughout this investigation. I wish to express my thanks to Dr. Henry A. Christian for making this work possible.

THE INFLUENCE OF DIGITALIS ON THE T WAVE OF THE HUMAN ELECTROCARDIOGRAM.*

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PLATES 59 TO 64.

The modifications in the form of the T wave of the electrocardiogram which we report in this communication were observed in the course of detailed studies on the action of digitalis in patients (1). It has been noticed frequently that the time when digitalis takes effect is a matter of uncertainty, so that in a given instance doubt is often entertained as to whether the drug itself is active, and if active, whether it is effective in producing a result. To be certain that the drug is effective, it has been considered necessary to administer it until an alteration in the rhythm of the heart occurs. In our treatment of patients we have, therefore, looked for evidences of altered heart rhythm to indicate that the heart is really digitalized. During the period of treatment, electrocardiograms were made almost daily, and often more than once a day. In examining these records we found the changes in the T wave which we describe in this report.

Other observers have published studies dealing with the effect of a number of agents, including digitalis, on the electrocardiogram. The only investigation dealing with this subject in human beings has been made by Nicolai and Simons (2). Seven ambulatory patients were given powdered digitalis, 0.3 gm. daily, for five days. In two women the treatment was not completed; in one normal man the T wave did not increase in height, but decreased instead (*"verschlechterte sich"*); and in the other four the T wave increased. They limited the taking of records to the first lead (right arm to left arm). Even if the

* A preliminary report was made to the Section on Medicine at the Seventeenth International Congress of Medicine, London, 1913, Section VI, 255-258. Received for publication, April 7, 1915.

dosage had been sufficient, and had been satisfactorily controlled, a comparison of their methods with ours would account for the difference in our results.

A number of experiments on animals have been published in which digitalis bodies, more especially strophanthin, were injected for the purpose of observing possible changes in the electrocardiogram under the influence of the drug. Straub, Selenin, Bickel and Tsividis, Bickel and Pawlow, and Rothberger and Winterberg have published such reports. In general all observers have detected an increase in size of the T wave under the influence of digitalis, like that described by Nicolai and Simons. Straub (3) described experiments in cats and rabbits, into the veins of which he injected strophanthin (Böhringer). So called therapeutic doses, the exact quantity of which is not given, caused no alterations in their electrocardiograms. When he gave toxic doses, that is to say, 0.3 mg., he obtained an increase in the elevation of the T waves in one experiment, the curve of which he reproduces. He obtained a multiplicity of abnormal ventricular complexes in the late stages of intoxication, but obviously the T waves in these forms cannot be compared with those in the control curves. In a later paper (4) he described experiments on the isolated frog heart, and showed that in the therapeutic stage the T wave increased in size, as in his mammalian experiments, while in the late toxic stages the T wave became negative. The changes in the late toxic stage in frogs are not like those in mammals, because the former do not develop abnormal ventricular complexes.

Selenin (5) found that the T wave increased in size when a digitalis body was given in therapeutic doses to dogs.

Bickel and Tsividis (6) injected digitalisatum Bürger into the ear veins of rabbits. Small and medium doses (1 c.c. per kg.) increased the size of the waves. Larger doses decreased the size of the waves, including the T wave, but they remained upward in direction.

In a later paper Bickel and Pawlow (7) injected crystalline strophanthin (Thoms) intravenously into dogs, and digistrophan, a combination of digitalis and strophanthin, into dogs and rabbits. They concluded that small doses increased the size of the T wave, whereas large doses decreased the size of all waves. They mention no instance of inversion. They found that the T wave tended to change less than the other waves. Other substances supposedly effectual on the heart, like cardiotoxin, a mixture of convallaria and caffeine sodium benzoate, were quite ineffectual.

Rothberger and Winterberg (8) found that small doses of strophanthin given to dogs had no effect on the shape of the electrocardiogram. When P and T waves were small as the result of the heart's having been isolated from the central nervous system, they report that digitalis restored them to their initial size. When they gave digitalis in progressively larger quantities, aside from alterations in the P, R, and S waves, the T wave became either positive or negative. In the experiments of all these investigators the results depend on the injection of single doses of the drug, the quantities being larger than those which are employed in therapeutics. The change in the electrocardiogram usually obtained is considered to be an increase in the elevation of the T wave. Straub alone seems to have regarded the change in sign in the T wave in his experiments on frogs as unusual and important.

The shape of the T wave has also been changed by the use of other means.

Muscarin, according to Samojloff (9, 10, 11) and Boruttau (12), alters not only its form, but in frogs changes the sign of the wave from positive to negative. After the alteration has taken place, both noticed that the application of atropin restores the initial direction of the wave. Mines (13) believed that spread from the site of application to the ventricular base was responsible for Samojloff's result and that muscarin does not alter the sign of the T wave except on direct application. After alteration has taken place, atropin relieves it. But in his figures 2 and 3 the sign of T changed from negative to positive. Here the heart was perfused with muscarin so that the possibility of local action is excluded. Samojloff and Mines (14, 15) both observed that electrical stimulation alone of the vagus nerve in frogs without the use of drugs produced an inversion of the T wave.

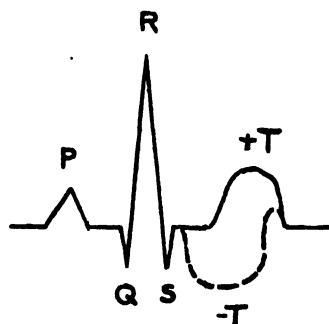
OBSERVATIONS.

In the curves of the patients under treatment with digitalis, attention was arrested by the fact that alterations in the T waves of the electrocardiograms occurred in a large number (30 times in 34 patients), and also by the fact that the change was detected before alterations in rhythm or conduction time had occurred and before symptoms referable to the gastro-intestinal tract disturbed the patients, except in five instances. The changes in the T wave consisted in a diminution in the height of the wave, and, finally, in an inversion (figure 1). This diminution in height to an isoelectric line and subsequent inversion of the wave are not the only changes which occurred. If the space from the end of R or S to the end of T is considered, additional alterations become evident. The first part of this interval, from R or S to T, is usually isoelectric or shows a slight upward slope. In place of these conditions may be substituted either an isoelectric period, if it is not isoelectric already, or a downward deflection, directly continuous with the end of R or S. The downward deflection is often carried to a point just short of where in the uninfluenced curve the T wave ends. At this time, then, the inverted wave passes above the base line to form an upward deflection, representing the terminal portion of the original wave. It is indicated in figure 1 D. Text-figure 1 shows this relation. The solid line indicates the outline of the initial curve; the dotted line, the alteration described. Samojloff (11) has drawn a similar diagram.¹ Another alteration which is sometimes seen is shown in text-figure 2. Here the downward deflection occurs, not in the

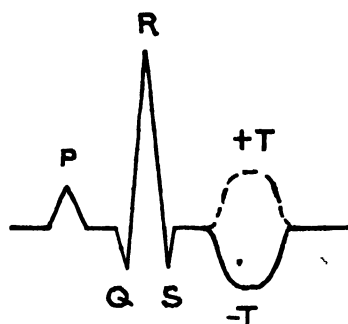
¹ Samojloff, *loc. cit.* (11), p. 481, figure 2 C.

early portion of the period, but in the later portion. Instead of a diphasic wave in which the upward deflection occupies the last portion, as in text-figure 1, it is in such instances in the first portion.

Other alterations beside those described have been seen when digitalis was administered. One was in a case of complete auriculo-ventricular dissociation. The observation in this patient was especially valuable because he showed, at different times, two quite differ-



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIGS. 1 and 2. The unbroken line represents the electrocardiogram, and shows the P, Q, R, S, and T waves. The broken line shows the outline the T wave assumes under the influence of digitalis.

ent electrocardiograms. One indicated an origin for the ventricular beats in the left side of the heart (figure 2); the other, in the right side (figure 3). In figure 2, taken when the ventricular beats were initiated on the left side, inverted T_1 ² became deeper under the influence of digitalis, inverted T_2 moved upward, and so did diphasic T_3 . When the ventricular beats were initiated on the right side (figure 3) diphasic T_1 became isoelectric, inverted T_2 turned upward, and inverted T_3 became shallow, almost isoelectric, and diphasic, while the completely restored curve resembled its control except that T_1 was a monophasic wave, replacing a diphasic wave in the initial curve. That the change was less far reaching in figure 3 than in figure 2 may have been due to the lesser amount of digitalis which had been given, 1.9 instead of 2.6 grams. These curves supply an instance of a change in the sign of T_2 and T_3 from negative to posi-

² T_1 represents the T wave in lead 1; T_2 in lead 2, etc.

tive under the influence of the drug, a change of sign in the reverse direction from that first described.

Still other types of change have been found. In a case, for instance, in which T_1 and T_2 were positive, and T_3 negative (figure 4), T_2 became negative, and T_3 more negative. In pathological hearts, such as these, the redistribution of muscle, resulting from hypertrophy and dilatation, doubtless determines a variety of alterations in the electrocardiogram under the influence of digitalis. We do not at the present time possess a sufficient number of instances of a given class to permit us to reduce the alterations in them to definite order. Originally it appeared to us that T_3 altered earlier than T_2 . Our later observations make us hesitate to state definitely whether the T wave in one lead is influenced earlier than in the others, or whether changes occur simultaneously in all. Recent interpretations of the electrocardiogram by Einthoven, Fahr, and de Waart show that simultaneous alterations may be anticipated, and our later observations indicate that this is so.

The instances so far described relate to cases in which the mechanism of the heart beat was normal. Similar alterations have, however, also been seen when the auricles were fibrillating (figure 5) and also when they fluttered. In curves of this type, recognition of altered T waves may occasionally be difficult, especially if the waves are not large, on account of the greater number of oscillations in the curve, due to the abnormal auricular activity.

We have pointed out that with five exceptions the modifications described were seen at a time when neither a change in rhythm or conduction time nor the onset of gastro-intestinal symptoms had taken place. An alteration in the curve may, therefore, be taken as a sign of the fact that an influence by digitalis is being exerted on the heart. The sign attains the greater importance on account of its appearance early after the beginning of the administration of the drug. We have detected changes in the T waves after an equivalent of 1.2 grams or even less of the dried leaves³ of digitalis has been given, that is to say, on the third day of administration, although

³ We have used digipuratum, a tablet of which represents 0.1 gm. of dried leaves. We usually give 0.4 gm. a day. The dose varies naturally with the patient's requirements. We have not estimated the size of the smallest dose capable of producing a change.

on several occasions we have seen the altered forms after thirty-six to forty-eight hours. On account of certain matters of technique, we have hesitated, in our earlier records, to recognize the onset of the alteration as early as it is quite apparent to us now that it occurs. The sign may, therefore, be looked on as one capable of detection earlier than the others, and as one indicating the fact that the drug is acting. Small changes measured in hundredths of seconds in the auriculoventricular conduction time occasionally occur as early as that noticed in the T wave, and when present may, of course, be employed as corroborating evidence of the fact that the drug is acting. The length of time required before the T wave is restored to its initial state varies. In the patient whose curves are reproduced in figure 6 twenty-two days elapsed before complete restoration occurred. In other cases it required only five days.

The influence of atropin on the T wave, after an alteration in it has become established, was tested more than forty times. Atropin sulphate was given either subcutaneously or intravenously in doses ranging from 0.9 to 1.8 milligrams, the size of the dose depending on the age of the patient and the method of administration. In every instance, the alteration which had taken place persisted after the drug was given. In several instances, when no change had been observed before atropin was injected, the characteristic modification appeared under the influence of the drug. Those alterations which atropin brought on or intensified almost always disappeared promptly; that is to say, before the next curve was taken, twenty-four hours later. Atropin alone, when no digitalis had been given, did not produce changes in the T wave. We have, however, an instance in which atropin lowered the isoelectric line between R and T slightly. Another modification in the outline of the digitalis curve occurs during the activity of atropin. The acceleration in rate which atropin causes is accompanied by a reduction in the time which elapses from the beginning of R (Q) to the end of T (figures 1 and 6 and text-figures 1 and 2). As a result of acceleration, the various elements which make up the ventricular electrocardiogram contract, but the essentials of the change which digitalis has brought about in the curve remain quite unaffected.

DISCUSSION.

In view of the fact that for human beings the form of the electrocardiogram is constant, and varies only because of distinct cause, the changes in it which have just been described must be looked upon as changes of significance. That they result from the action of digitalis must be concluded from the fact that they occur almost invariably when the drug is administered, and disappear more or less promptly when administration of the drug is discontinued. If the drug is given again to the same patient, after an interval during which the initial form of the electrocardiogram is restored, the same changes take place.

The interpretation of the changes in the T wave which have been described as occurring under the influence of digitalis presents difficulties, based on the interpretation of the formation of the electrocardiogram itself. If we adopt the current view, held by the earlier writers, Burdon-Sanderson and Page, Bayliss and Starling, and most of the later investigators, Einthoven, Samojloff, Garten, Boruttau, and Mines, that the cardiac action currents depend on electrical changes as an expression of muscular activity, then the changes in the T wave must be attributed to an alteration in muscular state under the influence of the drug. The fact that the T wave becomes negative indicates that the preponderance of electrical activity persists in the region near the apex of the heart longer than it does at the base; or, conversely, shorter at the base than at the apex. This is substantially the explanation that Samojloff and Boruttau adopt in explaining the action of vagus excitation and of muscarin. It opposes the view that persistence of electrical activity depends on a specific action directed toward a given part of the heart muscle, as Selenin supposed.⁴ Greater electrical activity near the apex, therefore, accounts for apex negativity, and the consequent inversion of the T wave. The variations in the alteration of the terminal part of the wave already described depend on the exact site of longer electrical activity. If electrical activity at the base outlasts that at the apex, a diphasic wave results, the first portion of which is directed downward, corresponding to greater activity at the apex, and the second phase ends in a short upward deflection, corresponding to

⁴ Selenin, *loc. cit.*, p. 154.

greater persistence of activity at the base (text-figure 1). Occasionally activity lasts longer, in the terminal phase of ventricular systole, at the apex, and this is shown by a diphasic wave, the direction of which in its first portion is upward, if the activity at the base is the greater, and in the latter portion downward (text-figure 2).

The observations of numerous pharmacologists on the action of digitalis bodies on the frog's and on mammalian hearts have demonstrated a diminished diastolic relaxation of the apical regions of the ventricles. In the later toxic stages of the action of the drug a systolic standstill of the apical regions is seen, while the base is still relaxing in diastole.⁵ The changes we have observed in the T wave during the action of digitalis on the human heart may be evidence of a similar condition of the apical portion of the ventricles.

A fact to which we have called attention in these observations necessitates a possible modification in the view that the effect of digitalis is, so far as its influence on the T wave is concerned, wholly muscular. We refer to the fact that, on occasion, when a heart is subjected to the influence of digitalis, no change or only a slight one, takes place in the outline of the T wave. If atropin is injected under these circumstances, the alteration in the T wave, which was expected but failed to appear, becomes manifest immediately; the T wave either becomes inverted or becomes more negative than it was. When the influence of atropin terminates, the T wave returns to the state before injection. The view taken that atropin acts on the terminals of the inhibitory nerves implies, in this connection, that these exert an influence on the alteration in the wave, besides that attributable to the action of digitalis on heart muscle. Their influence, stimulated perhaps by the action of digitalis, is exerted against the full expression of increased electrical activity on the part of a given muscle area. When atropin is given, the inhibitor power is removed, and the full influence of digitalis on the muscle is manifest.

⁵ This phenomenon is recorded by Cushny, A. R., *A Textbook of Pharmacology and Therapeutics, or the Action of Drugs in Health and Disease*, 4th edition, Philadelphia, 1906, 439. Asynchronous contractions of the frog's ventricle, due to strophanthus, are also described by Fraser, T. R., *On the Kombé Arrow-Poison (Strophanthus cuspidus, D.C.) of Africa*, *Jour. Anat. and Physiol.*, 1873, vii, 139.

These observations have yielded two-fold evidence bearing on the nature of the mechanism of digitalis action, considered from the point of view of whether this is muscular or nervous. We recall, in the first place, the difference between the effect of atropin on the T wave during digitalis, and during muscarin action. It has been shown that both drugs are capable of producing modifications in the T wave. The injection of atropin in the case of muscarin abolishes these, according to Samojloff and Boruttau, while in the case of digitalis the change persists or is intensified. The difference brought out between the two by the injection of atropin appears to depend on a difference in the site of their respective actions. The site of the action of muscarin on the cardiac inhibitors is well established;⁶ its effect is consequently abolished when atropin, which acts on the nerve terminals, is given. It follows from this that if the inversion of the T wave under the influence of digitalis is not relieved by the injection of atropin, that influence is exerted at a site distal to the nerve endings; that is to say, on the heart muscle. We have shown that the inhibitors can prevent the formation or the full development of inversion in the T wave because it sometimes does not appear until after atropin is given. This curious phenomenon is exerted in opposition to the obvious muscular digitalis effect, and represents what appears to be a new phase of the function of the nerves. The second observation relating to the mechanism of the action of digitalis to which we have referred shows that it has two separate and distinct effects on the heart. The first consists in an alteration in the mechanism of the heart beat, a function which has been abundantly proved, and the second in the alteration of the T wave we are describing. The effect of the injection of atropin indicates that these two alterations depend on two activities of digitalis, quite distinct from each other, for the altered mechanism is abolished, while the altered T wave either persists or is intensified (figure 6). We attribute the altered heart rhythm which atropin is able to relieve to the action of digitalis on the inhibitory mechanism. The altered T wave which atropin fails to restore to its initial

⁶ Recently the site of the action of muscarin has been reconsidered in papers by Straub, Samojloff, and Mines. Inasmuch as all agree that atropin has the effect mentioned, the argument advanced is considered valid.

state we attribute, as we have just shown, to the muscular action of digitalis. We assume for the purpose of this discussion that the effect of atropin is uniform; but we shall show in another place that this is true only within certain limits.

We wish finally to indicate the bearing that these observations have on the phenomenon of persistence of digitalis action. If alteration of the T wave depends on the action of digitalis, the time which elapses between the end of its administration and the return of the T wave to its initial state must be considered the measure of time during which digitalis is an active agent. The length of time required for restoration to the initial state has been seen to vary. In one observation it has been seen to take as little as five days; it has been observed to require as long as twenty-two days. The latter, if it is a common example of the duration of action, is a longer period for the drug to be active than is commonly supposed. Persistence of action may be compared with the duration of digitalis effect on rate in auricular fibrillation. In at least one such patient we have several times seen fourteen days elapse, after the administration of digitalis was stopped, before the ventricular rate began to rise. The statement (16) sometimes made that a second course of treatment with digitalis requires less of the drug to produce a given effect than the first is probably explained by our observation. For it is clear that if a second course is begun, before the first is completely terminated, a greater amount, equal to the residual quantity of the drug, must be present in the heart than had been expected.

SUMMARY.

It has been shown in this investigation that digitalis, administered orally to patients, can modify the T wave in the electrocardiogram. When the T wave in the initial curve is directed upward, the first change noticed is a lowering, and the final change is an inversion of the wave. It is not only the wave itself, but that portion of the curve between the end of R and the end of T which is involved. Instances in which the initial T waves have other than upright forms are described and their behavior under the influence of digitalis has been indicated. This influence of digitalis on the T wave may be

detected in thirty-six to forty-eight hours after the administration of digitalis has commenced; it may persist as long as twenty-two days after the administration has been stopped. Instances where it persisted only five days have been encountered. The unexpected length of duration of the sign probably explains why a second treatment with digitalis requires a smaller amount of the drug to produce the same effect, than the first.

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EXPLANATION OF PLATES.

In all figures divisions of the abscissæ equal 0.04 of a second; divisions of the ordinates equal 10^{-4} millivolts.

PLATE 59.

FIG. 1. A to H. The curves were taken from a patient who had a moderate grade of arteriosclerosis. A is the control. B, C, D, and E were made under the influence of digitalis; a few minutes before E atropin was injected. F and G show the gradual return to the form of the control. H substantially reproduces the control, A. The date to the left of each curve indicates when the curve was taken. The three usual leads (Einthoven) arranged in columns were obtained on each occasion. During the atropin test only lead 2 was made. The total amount of digitalis taken at the time each curve was made is given at the right.

PLATE 60.

FIG. 2. A to C. The curves were taken from a patient who had complete auriculoventricular dissociation. The three leads are arranged from above down. A is the control. B was taken after digitalis, 2.6 gm., was given. C substantially reproduces the control. The date on which each set of three curves was made is placed below, likewise the rate of the ventricles.

PLATE 61.

FIG. 3. A to D. These curves were taken from the same patient as those in figure 2. A is the control; B was taken under the influence of digitalis; C shows partial recovery; D substantially reproduces the form of the control curve.

PLATE 62.

FIG. 4. A to L. These curves were taken from a patient who had mitral stenosis. The leads are arranged in columns. The date when each curve was taken is given at the left. A is the control. B, C, and D were taken under the influence of digitalis; the amount taken when the curve was made is given at the right. E to K shows the gradual return to the control. J, a single instance, breaks the gradation. L substantially reproduces the control curve.

PLATE 63.

FIG. 5. A to C. These curves were taken from a patient whose auricles were fibrillating. The leads are arranged as in figure 2. A was taken after digitalis, 3.2 gm., had been given. B is the control. C was taken after digitalis, 3.6 gm., had been given.

PLATE 64.

FIG. 6. A to C. These curves were taken from a patient who has mitral stenosis. The leads are arranged as in figure 2. A is the control. B was taken after digitalis, 2.5 gm., was given. The altered cardiac mechanism is described in the text. B₁ was made shortly after B, when atropin, 1.0 mg., had been injected. C substantially reproduces the control curve.

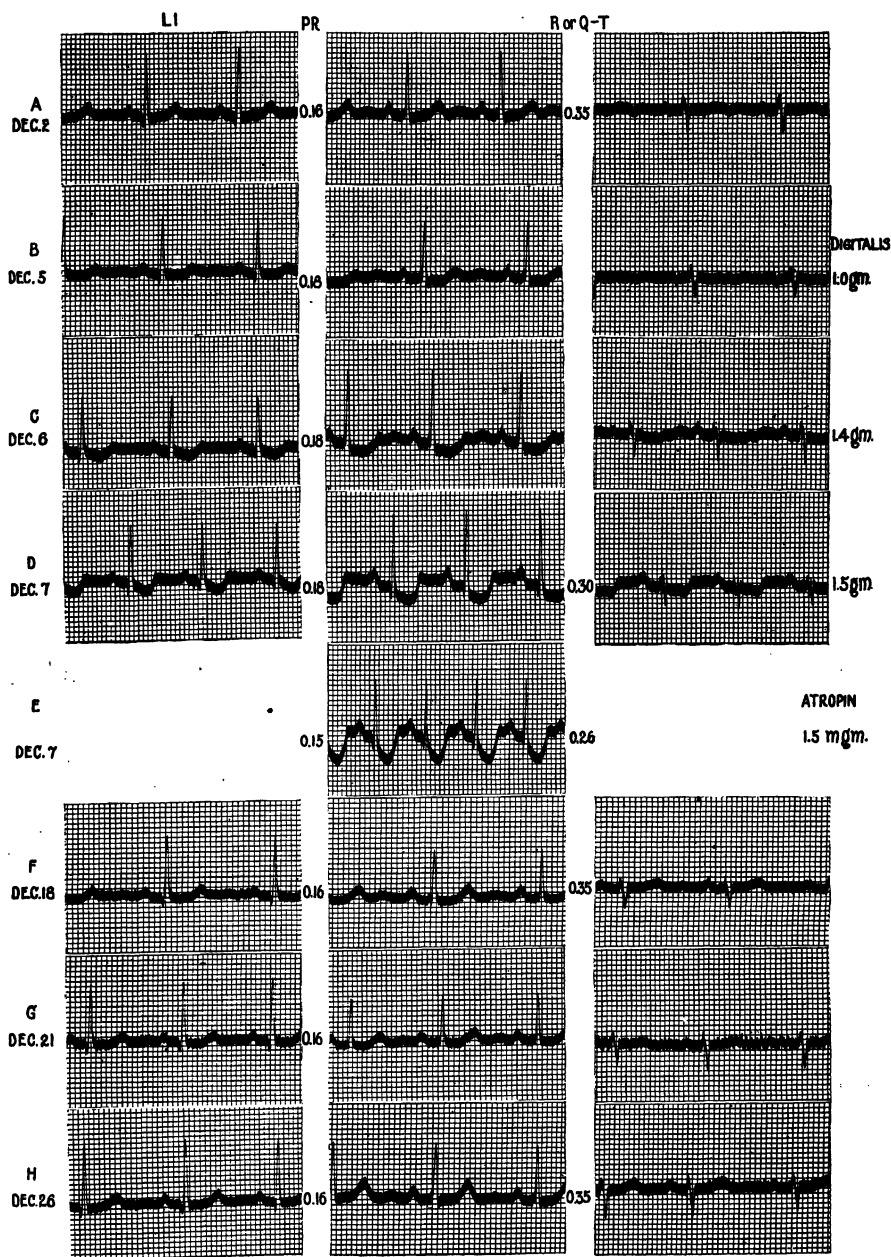


FIG. 1.
(Cohn, Fraser, and Jamieson: Influence of Digitalis on Human Electrocardiogram.)

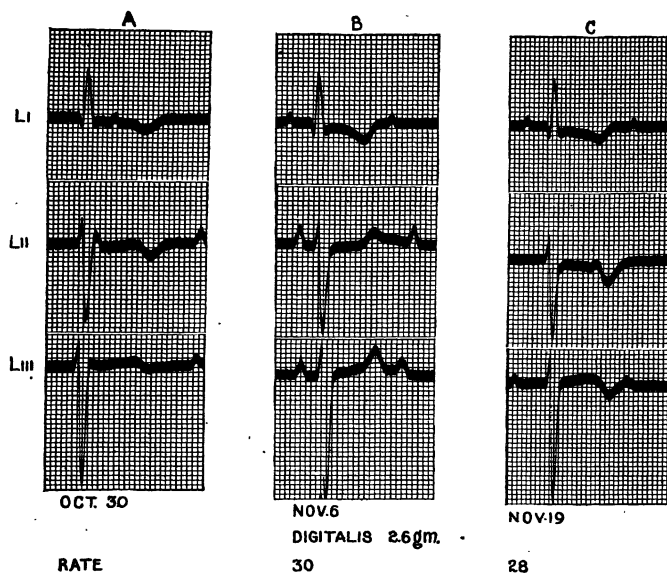


FIG. 2.
(Cohn, Fraser, and Jamieson: Influence of Digitalis on Human Electrocardiogram.)

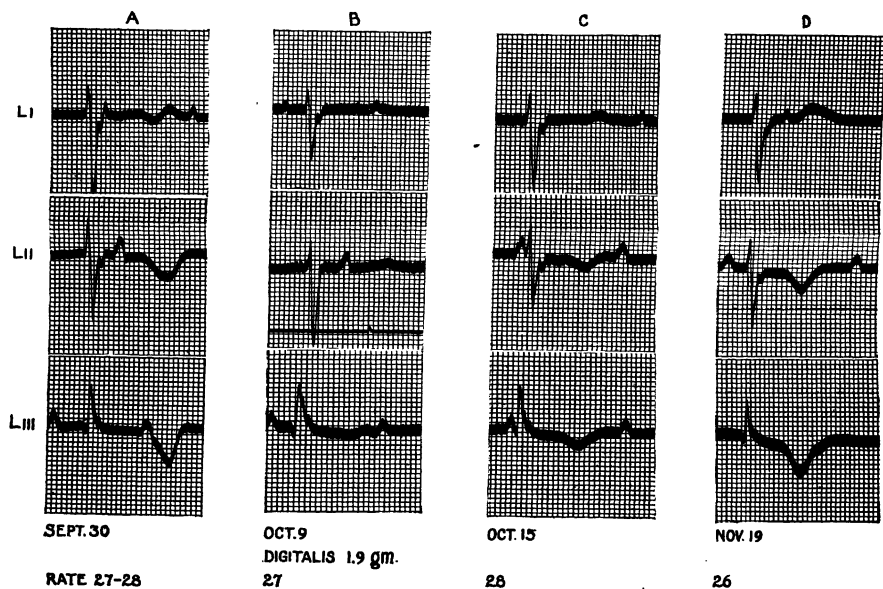


FIG. 3.
(Cohn, Fraser, and Jamieson: Influence of Digitalis on Human Electrocardiogram.)

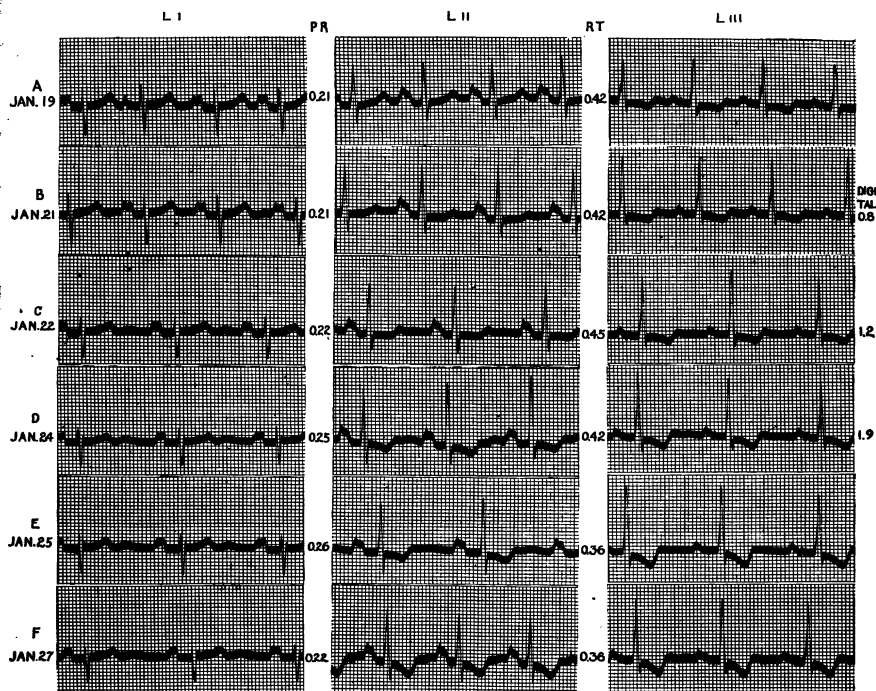


FIG. 4.

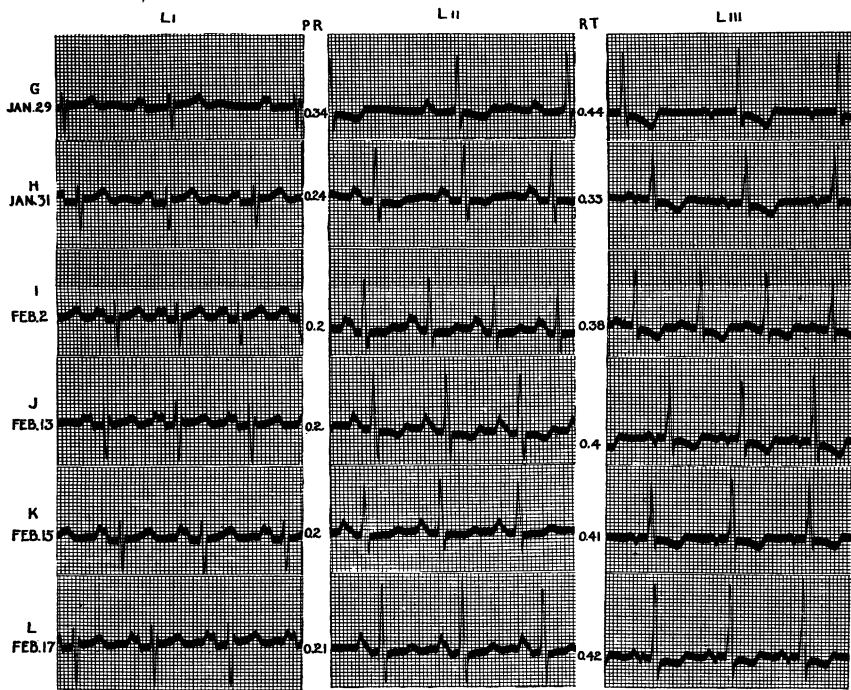


FIG. 4A.
(Cohn, Fraser, and Jamieson: Influence of Digitalis on Human Electrocardiogram.)

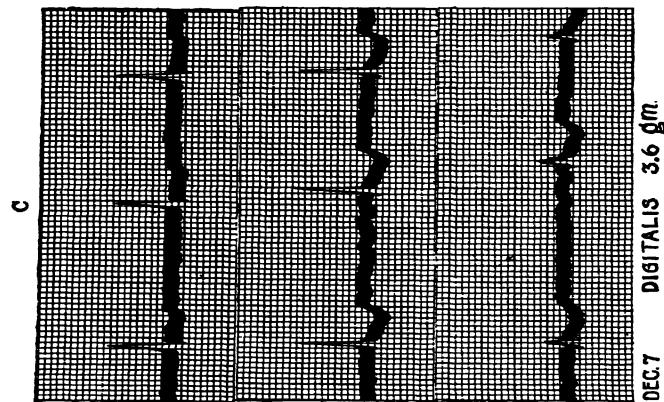
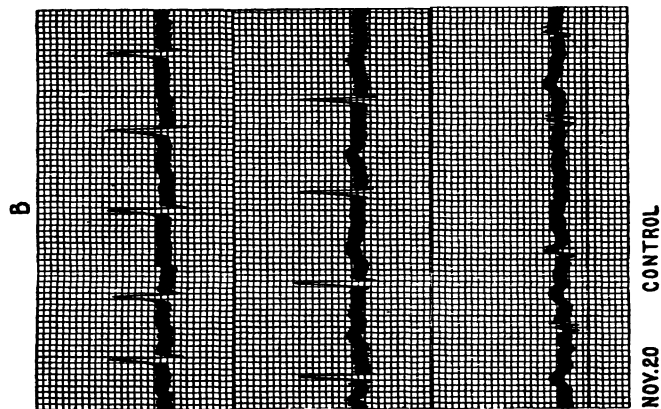
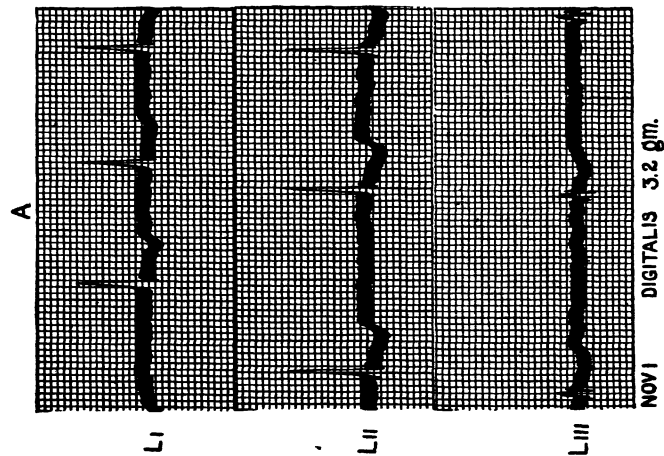
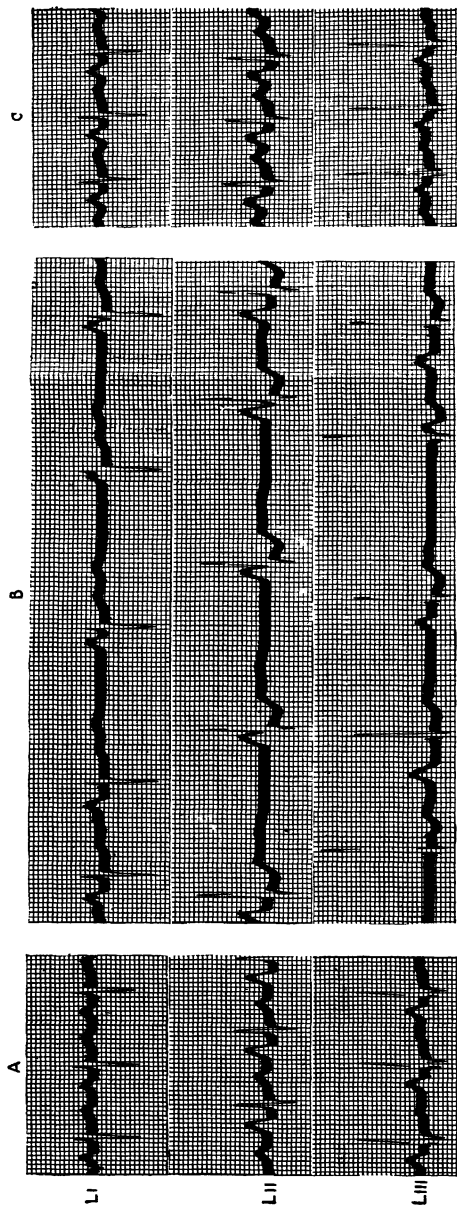


FIG. 5.

(Cohn, Frazer, and Jamieson: Influence of Digitalis on Human Electrocardiogram.)



0.2

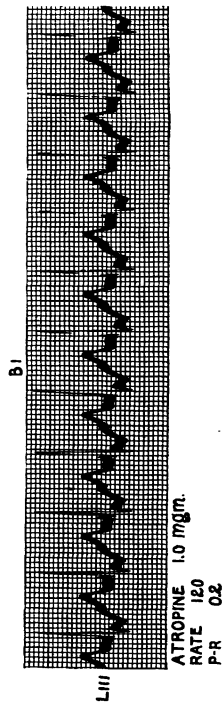


FIG. 6.

(Cohn, Fraser, and Jamieson: Influence of Digitalis on Human Electrocardiogram.)

FORMS OF NITROGEN IN THE STOOLS OF INFANTS.*

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It has for some time been recognized that the *amount* of nitrogen lost in the stools of infants is rarely of great importance from the point of view of nutrition. The percentage of the intake which escapes absorption seldom constitutes a serious loss. It seemed possible, however, that significant information concerning the manner in which the alimentary tract handles the food proteins under varying conditions might be obtained by a study of the relative *proportions* of the fecal nitrogen which are excreted in the form of protein, and of amino acids and ammonia, the two chief final split products of protein. When proteins are digested under aseptic conditions by the intestinal enzymes, trypsin and erepsin, amino acids are the main products, and the amount of ammonia is relatively small. Putrefactive bacteria, on the other hand, are capable of changing a large percentage of the nitrogen of proteins and amino acids into ammonia. Consequently an increase of ammonia in the fecal nitrogen at the expense of the amino acids might serve as an indication of the activity of putrefactive bacteria. A relatively high proportion of amino acids, on the other hand, might indicate incomplete absorption of the protein digestion products, despite complete digestion and absence of putrefaction.

Our results failed to show any constant relations between the clinical condition of the patients and the distribution of the fecal nitrogen among the three forms discussed above. The data obtained are, however, not without interest in their revelation of the form in which the unabsorbed nitrogen leaves the alimentary canal,

* The observations reported here were begun in the fall of 1913, and have since been continued at intervals as cases suitable for study have presented themselves.

particularly as previous information on this point has been very meager.

Methods.

The method of collection made it almost impossible for a urine contamination to occur without our knowledge. The stools were used either when freshly voided or were placed at once in an ice-box to avoid any further decomposition. Forty to 50 gm. of the fresh feces were emulsified in water and diluted to 200 c.c. The mixture was made homogeneous by grinding in a mortar and aliquot portions were taken for the following determinations.

Total Nitrogen.—Five c.c. were used for a Kjeldahl determination.

Ammonia.—Ten c.c. were placed in a 100 c.c. test tube, 4 to 5 gm. of potassium carbonate, with 2 drops of octyl alcohol to prevent foam, were added, and the ammonia was determined by aeration into fiftieth-normal acid by Folin's method.

Urea.—Ten c.c. were placed in a 100 c.c. test tube with 5 c.c. of 0.6 per cent. KH_2PO_4 and 1 c.c. of 10 per cent. "Arlco-urease."¹ After fifteen minutes, allowed for hydrolysis of the urea, from 8 to 10 gm. of potassium carbonate were added and the ammonia aerated into fiftieth-normal acid. The increase in ammonia resulting from the action of the urease represents the urea nitrogen. The accuracy of the method under the conditions described was shown by control analyses on ten specimens of feces. Known amounts of urea were added to measured portions of the emulsified feces. In every case the determinations showed an increase in urea equal to the amount added.

Free Amino Acid Nitrogen.—To 100 c.c. of emulsion about 0.5 gm. of charcoal was added, and the solution was filtered without washing the residue. The volume of the filtrate was measured, and to it 10 per cent. metaphosphoric acid was added until all the protein was precipitated. The volume of metaphosphoric acid solution required was 15 c.c. or less. After a half hour had been allowed for complete precipitation of the proteins, the mixture was made up to a convenient volume, made alkaline by addition of powdered calcium

1. Van Slyke and Cullen: A Permanent Preparation of Urease and Its Use in the Determination of Urea, Jour. Biol. Chem., 1914, xix, 211.

hydroxid, and filtered. The filtrate was free from protein, as indicated by the biuret test. The filtrate was measured and evaporated to dryness on a water bath. During evaporation the solution, made alkaline by the above mentioned addition of lime, entirely loses its ammonia. The foregoing treatment therefore frees the solution of both protein and ammonia. The dry residue was taken up with 1 c.c. of 50 per cent. acetic acid and washed into a 10 c.c. measuring flask with small portions of water. Two c.c. portions were used for determination of amino nitrogen by the nitrous acid method.²

Total Amino Acid Nitrogen after Hydrolysis of the Proteins.—Ten c.c. of the feces suspension were mixed with 10 c.c. of concentrated hydrochloric acid, and the solution, in a loosely stoppered tube, was immersed in a water bath at 100° for twenty-four hours. This assures practically complete hydrolysis of the proteins present.³ The solution was evaporated to dryness on the water bath to remove the hydrochloric acid. The residue was taken up with water, made alkaline with calcium hydroxid, and filtered. The alkaline filtrate was concentrated to dryness to remove the ammonia. It was then redissolved with the help of 1 c.c. of 50 per cent. acetic acid; the solution was made up to 25 c.c., and 2 c.c. portions were taken for determination of amino nitrogen.

Acidity.—An aliquot part, usually 25 c.c., of the suspension of feces was titrated with tenth-normal sodium hydroxid, phenolphthalein being used as indicator. The method is, of course, a crude one, but yields comparable results.

DISCUSSION OF RESULTS.

As the accompanying tables show, the proportion of the total nitrogen in the form of NH_2 of the *free amino acids* varies greatly, the range being from 1.8 per cent. to about ten times as much—17.6 per cent. The present data show no factor in the diet or condition of the patients which has an entirely constant effect on the proportion of nitrogen passing unabsorbed through the intestine either as ammonia or free amino acids. The more nearly normal

2. Van Slyke: Jour. Biol. Chem., 1913, xvi, 121.

3. Van Slyke: Conditions for the Complete Hydrolysis of Proteins, Jour. Biol. Chem., 1912, xii, 295.

TABLE 1.—Forms of

No.	Age in mos.	Condition	Food†
1	5½	Convalescent from diarrhea and wasting.	2-4-1.8 with barley flour.....
2	4	Normal.....	Breast milk.....
3	18½	Drowsy and feverish, improving very slightly.	Soft diet; egg, bread, cereal, sauce, milk.
4	5½	Convalescent from diarrhea and wasting.	2-4-1.8 with barley flour, and malt soup extract.
5	8½	Convalescent from diarrhea.....	Protein milk.....
6	19	Feverish, with slight cough.....	3-3.4-2.8 and cereal.....
7	11	Nearly normal after acute indigestion.	Protein milk with barley flour.....
8	7	Convalescent from enterocolitis..	1.4-1.75-1.2 (from condensed milk, boiled with sodium bicarbonate) with cane sugar and barley flour.
9	6½	Slowly convalescent from wasting, diarrhea and vomiting.	1.6-2-1.4 with barley flour (from condensed milk cooked with sodium bicarbonate).
10	5½	Convalescent from diarrhea and wasting.	2-4-1.8 with barley flour and malt soup extract.
11	14½	Slowly convalescent from diarrhea and wasting.	Protein milk.....
12	7	Convalescent from diarrhea.....	Protein milk with barley flour and malt soup extract.
13	1	Congenital malformation of heart.	1.4-1.5-1.2, from condensed milk and boiled with sodium bicarbonate.
14	6½	Convalescent from diarrhea and vomiting.	Protein milk with barley flour. Breast milk X3.
15	5	Marasmus, gaining slightly.....	Breast milk.....
16	3	Marasmus, gaining slightly.....	Breast milk.....
17	3	Marasmus, gaining slightly.....	Breast milk.....
18	5½	Convalescent from diarrhea and wasting.	2-4-1.8 with barley flour and malt soup extract.
19	5½	Convalescent from diarrhea and wasting.	2-4-1.8 with barley flour and malt soup extract.
20	7	Improving from diarrhea.....	Protein milk with barley flour and malt soup extract.
21	5½	Convalescent from diarrhea and wasting.	2-4-1.8 with barley flour and malt soup extract.
22	25½	Suspected tuberculosis. Gaining weight.	House diet: scraped meat, potato, bread, cereal, sauce, milk.
23	1½	Gaining slightly from wasting and vomiting.	1.4-1.75-1.2 with barley flour (from condensed milk and boiled with sodium bicarbonate).
24	6½	Slowly convalescent from wasting, diarrhea and vomiting.	1.6-2-1.4 changed to 1.8-2.5-1.6 with malt soup extract.

* The cases are arranged in order corresponding to the free NH₃ figures, the lowest being given first.

† The figures for the food formulas are given in the order, per cent. of fat, per cent. of milk sugar, per cent. of protein.

*Nitrogen in Infants' Stools.**

Stools	Gm. Total N, per Gm. Dried Wt.	Per Cent. of Total N. as			Acidity c.c. N/10 NaOH
		Free NH ₃	NH ₃ After Hy- drolysis	Free NH ₃	
Formed, hard and dry	.071	1.8	46.9	8.1	0
Watery in part; part pasty, greenish, sour. Soft curds.	.046	2.7	49.7	5.2	15.16
Constipated, formed and hard	.055	2.9	55.1	7.7	1.84
Mostly formed, hard and soapy in part	.054	3.3	49.7	3.6	2.57
Formed. Traces of mucus	.058	3.4	52.4	10.0	3.60
Constipated, formed and hard	.064	3.7	52.0	18.8	3.05
Part formed, in part smooth and pasty	(.05)	4.0		12.0	
Mostly formed, part pasty and smooth	(.05)	4.0		13.0	2.70
Very watery. Mucus and soft curds	.046	4.6	50.4	10.6	1.88
Mostly formed, soft and granular	.073	4.8	43.4	6.9	3.13
Part formed, part pasty. Large curds	.049	4.9	51.8	27.9	2.49
Part watery, part pasty, fermentative, acid. Mucus and curds.	.066	5.2	34.9		4.65
Very watery. Mucus and large soft curds	.11	5.8	25.3	9.7	11.28
Watery, large soft curds	.059	5.8	31.6	37.3	11.75
Part formed, part thin, pasty	.046	6.2	50.6	7.8	14.59
Watery, greenish, sour. Fat curds	.065	6.3	47.6	5.9	9.65
Watery, greenish, sour. Fat curds and mucus	.059	6.4	47.4	3.8	10.61
Watery and soft pasty, sour. Large soft curds	.051	6.6	47.7	7.6	7.95
Part formed, granular. Fine white curds	.058	6.8	52.5	4.3	3.97
Part formed, part thin, pasty, acid, fermentative, mucus.	.050	6.8			9.82
Soft, pasty, granular. Soft curds	.051	7.2	50.0	5.2	7.92
Thin pasty, oily-appearing	.035	7.6	47.8	7.0	12.60
Watery, full of fine white curds. Mucus	.052	7.7	36.9	23.1	5.15
Part formed, part watery. Soft curds and mucus	.071	8.0	41.8	29.2	.66

TABLE 1.—

No.	Age in mos.	Condition	Food†
25	5½	Convalescent from diarrhea and wasting.	2-4-1.8 with barley flour and malt soup extract.
26	4½	Improving from diarrhea	Protein milk with cane sugar
27	19½	Convalescent from bronchopneumonia.	Milk, two parts: water, one part
28	19½	Convalescent from bronchopneumonia.	Soft diet; milk, egg, bread, sauce, cereal.
29	8	Convalescent from diarrhea	1.4-1.75-1.2 from condensed milk and boiled with sodium bicarbonate and barley flour. Breast milk X4.
30	12	Convalescent from pneumonia	3-5-2.8 with beef juice, cereal, orange juice and sweet wine of iron.
31	7½	Improving slightly from diarrhea and vomiting.	1.8-2.25-1.6 from condensed milk and boiled with sodium bicarbonate and cane sugar and barley.
32	12	Convalescent from pneumonia	3-5-2.8 with beef juice, cereal, orange juice and sweet wine of iron.
33	12	Convalescent from pneumonia	3-5-2.8 with beef juice, cereal, orange juice and sweet wine of iron.
34	5	Marasmus improving	Same as No. 35
35	5½	Convalescent from bronchopneumonia.	1-5-2
36	16½	Physically normal, mentally deficient.	2.6-5-2.4 and cereal

babies showed low amino acid nitrogen, however, and those suffering from diarrhea generally showed the higher figures. None of the three breast-fed infants was among those eliminating a large proportion of amino acid nitrogen, and the only baby in the series that was both normal and breast-fed showed next to the lowest proportion of amino acid nitrogen in the feces. In the case that was followed for seven successive days (Table 2) it was noted that when the stools became loose the proportion of amino acids was increased, and vice versa. As a rule, to which there are exceptions, it appears that in the other cases also the more watery the stools the larger is the proportion of their nitrogen in the form of amino acids. Apparently the quicker passage through the alimentary tract results in a less complete absorption of the final products of protein digestion, viz., the amino acids.

In contrast to the wide variations above mentioned in the nitrogen of the free amino acids, the nitrogen of the *amino acids present after*

Concluded.

Stools	Gm. Total N. per Gm. Dried Wt.	Per Cent. of Total N. as			Acidity c.c. N/10 NaOH
		Free NH ₃	NH ₃ After Hydrolysis	Free NH ₃	
Part formed, granular. Fine white curds.	.053	8.4	49.0	4.7	3.74
Porridge-like, greenish.	.029	9.0	50.4	19.5	7.37
Mostly formed. Mucus.	(.05)	9.0		13.0	2.60
Pasty, granular, mucus.	.057	9.6	48.8	15.0	8.20
Watery, greenish, soft curds and mucus.	.065	10.2	55.5	7.6	9.30
Watery, acid, foul, with small and large hard curds.	.052	10.7	41.4	12.8	7.59
Mostly watery, granular, with fine white curds.	.049	13.3	52.6	8.8	7.48
Very watery, greenish, with large, hard curds, soft curds and mucus.	.054	13.6	50.5	12.6	10.15
Same as in No. 33.	.059	13.8	45.6	9.0	10.72
Part formed, partly thin, pasty, fermentative, mucus.	.058	14.3	46.4	17.2	9.67
Watery, greenish, small soft curds.	(.05)	16.0		8.0	2.80
Constipated, part formed and rather hard, part thin and smooth.	.038	17.6	44.1	9.2	1.30

hydrolysis of the protein was quite constant at from 43 to 55 per cent. of the total nitrogen. In a few exceptions this percentage was necessarily lower because the ammonia was so high. Most animal proteins when hydrolyzed show on the average about 75 per cent. of their nitrogen as free NH₂, the other 25 per cent. being amid nitrogen and the nitrogen of the amino acids, prolin, oxyprolin, tryptophan, arginin, and histidin, which have all or part of their nitrogen in forms other than NH₂ groups.⁴ Accordingly, if we multiply by 1.33 the amount of amino nitrogen found after acid hydrolysis of a mixture containing either animal proteins or their digestion products, we obtain approximately the amount of nitrogen present as protein and proteolytic products. Applying this factor (1.33) to our results for amino nitrogen after hydrolysis, we find that the feces on the average contain from 60 to 70 per cent. of their nitrogen in these forms. The free amino acid nitrogen alone constitutes

4. Van Slyke: Jour. Biol. Chem., 1911, x, 15; 1912, xii, 295.

TABLE 2.—Cases

Case	Condition	Date	Food
E. V., 5½ mos.	Convalescent from diarrhea; doing well. Weight fluctuating, but rising on whole.	November 6 ..	2-4-1.8 with barley and malt soup extract.
		November 7 ..	2-4-1.8 with barley and malt soup extract.
		November 8 ..	2-4-1.8 with barley and malt soup extract.
		November 9 ..	2-4-1.8 with barley and malt soup extract.
		November 10 ..	2-4-1.8 with barley.....
		November 11 ..	2-4-1.8 with barley.....
		November 12 ..	2-4-1.8 with barley.....
E. W., 12 mos.	Convalescent from pneumonia. Gaining weight.	December 19 ..	3-5-2.8 with beef juice and cereal.....
		December 20 ..	3-5-2.8 with beef juice and cereal.....
		December 21 ..	3-5-2.8 with beef juice and cereal.....

TABLE 3.—

Case	Condition	Date	Food
G. R., 4 mos....	Normal.....	December 22....	Breast-fed.....
A. R., 5 mos....	Marasmus, gaining slightly.....	January 29.....	Breast-fed.....
J. I., 3 mos....	Marasmus, gaining slightly.....	Jan. 30-Feb. 2...	Breast-fed.....
		February 2-5....	Breast-fed.....

from 2.4 to 24 per cent. of the total, while proteins, and possibly higher intermediate products make up the balance.

The *urea* determinations were not performed on the same stools as the other determinations, for the reason that when the latter were done the urea method had not been entirely perfected. We have, however, determined the proportion of urea nitrogen in a separate lot of stools and present the results in table 4. They show that the proportion of the total nitrogen in the form of urea varies between 9 and 5.6 per cent. Nineteen out of the 25 stools contained no urea at all. That its absence was due to bacterial transformation of urea into ammonia appears unlikely, because some of

Followed on Successive Days.

Stools	Gm. Total N. Per Gm. Dried Wt.	Per Cent. of Total N. as			Acidity c.c. N/10 NaOH
		Free NH ₃	NH ₃ After Hydrolysis	Free NH ₃	
Part formed, granular. Fine white curds	.053	8.4	49.0	4.7	3.74
Part formed, granular. Fine white curds	.058	6.8	52.5	4.3	3.97
Mostly formed	.054	3.3	49.7	3.6	2.57
Softer	.051	7.2	50.0	5.2	7.92
Softer, large soft curds	.051	6.6	47.7	7.6	7.95
Mostly formed again, though soft	.073	4.8	43.4	6.9	3.13
Entirely formed; hard and soapy	.071	1.8	46.9	8.1	0
Watery; curds, acid, mucus	.052	10.7	41.4	12.8	7.59
Watery; curds, acid, mucus	.059	13.8	45.6	9.0	10.72
Watery; curds, acid, mucus	.054	13.6	50.5	12.6	10.15

Breast-Fed Infants.

Stools	Gm. Total N. Per Gm. Dried Wt.	Per Cent. of Total N. as			Acidity c.c. N/10 NaOH
		Free NH ₃	NH ₃ After Hydrolysis	Free NH ₃	
Watery, soft curds, yellow-green	.046	2.7	49.7	5.2	15.16
Partly formed	.046	6.2	50.6	7.8	14.59
Watery, fat curds	.065	6.3	47.6	5.9	9.65
Watery, fat curds	.059	6.4	47.4	3.8	10.61

the stools which contained no urea also contained but little ammonia. It should be stated that unless the separation between stools and urine is most carefully supervised, a high urea figure must be questioned.

The ammonia varies between 4 and 37 per cent. of the total fecal nitrogen. It usually equals or exceeds the amino acid nitrogen. This fact gives a definite basis for the accepted opinion that the fecal ammonia is of bacterial origin. When animal proteins, such as casein, are split by non-bacterial agents, such as acids, or aseptic digestion with trypsin, the ammonia nitrogen is less than one-seventh the free NH₃ nitrogen.

TABLE 4.
Urea Determinations.

Name	Food	Description of Stool	Total N, Per Gm., Dried Wt.	Ammo- nia N, Per Gm., Dried Wt.	Ammo- nia N, Per Cent. of Total N	Urea N, Per Gm., Dried Wt.	Urea N, Per Cent. of Total N.
J. D., 6 mos.	1.6-4-1.4 with barley	Soft, pasty	.0390	.00054	1.4	0	0
V. C., 8 mos.	1.8-2.2-2 with barley	Watery	.0302	.00062	2.0	.00033	1.1
F. B., 5 mos.	Protein milk with rice flour and cane sugar.	Formed	.0375	.00130	3.6	0	0
F. S., 7 mos.	2-4-1.8 with barley	Part formed, part watery	.0396	.00060	1.4	0	0
A. A., 7 mos.	1.6-5-2 with rice flour	Mostly watery	.0488	.00055	1.1	0	0
Ach. A., 7 mos.	Protein milk with barley	Watery and pasty	.0493	.00100	2.2	0	0
A. R., 8 mos.	Protein milk with barley and beef juice.	Mostly formed	.0371	.00080	2.2	0	0
J. F., 9 mos.	2-5-1.8	Part formed, part pasty	.0373	.00070	1.8	0	0
J. F., 9 mos.	2.6-5-2.4	Formed	.0445	.00170	3.8	0	0
M. R., 4 mos.	1.8-6-2.4 with barley	Soft, pasty, green, mucus	.0682	.00429	6.3	.0011	1.6
N. M., 7 mos.	Protein milk with barley	Pasty, greenish-brown, foul; mucus	.0608	.00433	7.1	0	0
N. M.	Protein milk, 5 feedings; 2.6-5-2.4 with barley, 2 feedings.	Soft, pasty and watery, pale yellow	.0632	.00458	7.3	0	0
S. J., 9 mos.	Protein milk with barley and cane sugar, 5 feedings; breast milk, 2 feedings.	Mostly soft pasty, part watery, may be urine.	.0573	.00609	10.6	.00322	5.6
R. A'H., 5 mos.	Protein milk with barley and cane sugar, 5 feedings; breast milk, 2 feedings.	Formed, but soft; mucus and bean curds.	.0403	.00307	7.6	0	0
P. K., 4 mos.	2.8-5-2.6	Formed, pale yellow, part hard	.0367	.00198	5.4	0	0
M. L., 17 mos.	Soft diet	Watery, pale yellow, with curds and mucus.	.0453	.00487	10.7	.00117	2.6
W. C., 3 mos.	Protein milk with barley and cane sugar.	Formed but soft, pale; bean curds	.0504	.00383	7.6	0	0
A. H., 2 mos.	1.6-5-2.0	Part formed, part watery; mucus	.0737	.00392	5.3	0	0
L. A., 4 mos.	Breast milk; one feeding of protein milk; with olive oil.	Pasty, smooth; mucus	.0126	.00724	7.1	0	0
R. P., 5 mos.	2-5-1.8 with barley; breast milk, two feedings.	Very watery with fat curds and mucus.	.0183	.00395	21.6	.00103	5.6
C. S., 3 mos.	1.8-5-1.6, four feedings; breast milk, three feedings.	Very watery; oil floating on surface, bright yellow.	.0513	.00236	4.6	0	0
E. K., 6 mos.	1.2-4-2.2	Part pasty, part watery; many fat curds	.0462	.00179	3.9	0	0
		Pasty; portion formed and hard; mucus	.0386	.00351	9.1	.00074	1.9
		Part formed, constipated; part pasty, dark brown, smooth.					

No relation is perceptible between the proportion of ammonia and the condition of the patients or the nature of the stools. The amount of free amino acid nitrogen also bears no evident relation to the ammonia or to the acidity of the stools. The figures for each vary independently.

Nor does the acidity of the stools bear any observable relation to the ammonia. From the theory that fermentive and putrefactive bacteria are antagonistic, one would expect low ammonia when the acidity is high, and vice versa. It will be seen, however, that while this relationship exists in some cases, in others both ammonia and acidity are low, and in still others both are fairly high. In a few cases, such as No. 25, where a high ammonia content is accompanied by a low acidity, calculation indicates that the lack of acidity is due to the neutralization of acid by the unusual amount of ammonia, rather than to lack of acid formation.

SUMMARY.

Of the total nitrogen of infants' feces, from 50 to 70 per cent. was found as proteins and amino acids, 2.4 to 24 per cent. as free amino acids, and from 3 to 37 per cent. as ammonia. Usually, though not invariably, the higher percentages of amino acid nitrogen were found in the looser stools, quicker passage through the intestine resulting in somewhat less complete absorption of these protein digestion products.

The results show no evidence of antagonism between the processes of acid and ammonia formation respectively in the intestine.

Urea was certainly absent in three-fourths of the stools examined, and in the other fourth formed only from 1 to 5.6 per cent. of the total nitrogen. Despite the care taken to keep the feces free from urine, we do not feel certain that at least a part of the few positive results may not have been due to the admixture of traces of urine. As some even of the watery stools contained no urea, it appears improbable that the intestine is capable of rendering significant assistance to the kidney, at least in the excretion of urea.

A METHOD FOR THE DETERMINATION OF CHLORIDES IN SMALL AMOUNTS OF BODY FLUIDS.¹

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Previous methods for the estimation of chlorides in blood and plasma have required at least 10 cc. of material in order that the error might fall within 1 per cent of the total amount present. Bang's micro-method² requires only 100 mg. of blood, but it is hardly accurate to within 5 per cent.

The necessity for frequent chloride determinations on the plasma of individual patients, made simultaneously with other determinations on the same blood samples, led us to seek an accurate method of titrating small amounts of chlorides. As a result, we have recently published³ an iodometric method for determination of halides in general which permits the titration of as little as 5 cc. of $\frac{N}{10}$ halide solution with an accuracy of 1 part per 1000.

The present paper gives the conditions for the application of this method to body fluids. As the concentration of chloride in blood plasma is about $\frac{N}{10}$, it is possible to titrate the amount in 0.5 cc. with an accuracy of 1 part per 100. In order to leave a margin of safety we usually take more material, but the agreement between duplicates is then, as a rule, correspondingly closer than the 1 per cent accuracy which is required. The titration is also applicable to urine, ascitic fluid, pleural exudates, gastric juice, etc.

¹ A preliminary report of this method was published in the *Proc. Soc. Exper. Biol. and Med.*, xii, p. 93, 1915.

² I. Bang: *Biochem. Ztschr.*, xlix, p. 19, 1913.

³ F. C. McLean and D. D. Van Slyke: *Jour. Am. Chem. Soc.*, xxxvii, p. 1128, 1915.

Description of the Method.

The determination requires two steps: (1) removal of proteins, and (2) titration of chlorides.

Coagulation of Proteins.—Removal of the proteins may be accomplished in two ways, by coagulation or by ignition. Results are identical by both methods, but coagulation is the simpler and is the only means of deproteinizing which we have used in routine work. For coagulation 2 cc. of oxalated plasma (1 cc. may be used if material is limited) are drawn into a 2 cc. pipette which has been calibrated to contain 2 ± 0.005 cc. From the pipette the plasma is run into a 20 cc. stoppered volumetric flask which contains 10 cc. of a 10 per cent magnesium sulphate solution. The pipette is rinsed twice by drawing up into it the solution from the flask. Two drops of 50 per cent acetic acid are added, the flask is filled to the mark with water, the contents are mixed by inverting the flask, and heated in a bath to 100° for ten minutes. By keeping the stopper loosely in place evaporation is prevented, and when cool the contents return to their original volume. Ten minutes on the steam bath are sufficient to coagulate the albumin and to distribute the chlorides evenly between the fluid and precipitated albumin.⁴ A shorter period of heating may give low values in the filtrate, apparently due to a mechanical carrying down of chloride by protein, but at the end of ten minutes the filtrate contains its proper amount of chloride. The flask is then allowed to cool, and the contents are poured onto about 0.3 gram of blood charcoal in a small beaker, and mixed. (Merck's "Blood Charcoal, Reagent," purified by acid and free from chloride is used. No other form of charcoal has been found to be of service.) After a few minutes the liquid is filtered through a dry folded filter, and a water-clear filtrate obtained. Occasionally a small amount of charcoal passes through with the first few drops of filtrate, but this can be poured back through the same filter, and the filtrate then obtained is absolutely clear and colorless.

Titration of the Protein-Free Filtrate.—In brief, the chlorides are

⁴ The method of coagulation is similar to that of Larsson (*Biochem. Ztschr.*, xlix, p. 479, 1913), but much more rapid. Larsson coagulates at half volume, then dilutes after coagulation, and consequently has to wait two hours for the chloride to diffuse evenly through the coagulum.

precipitated in the presence of nitric acid by standard silver nitrate solution, the silver chloride is removed by filtration, and the excess silver titrated with standard potassium iodide. The titration is performed in the presence of nitrous acid and starch, so that the first drop of iodide in excess of the silver present is changed to free iodine and gives the blue starch-iodine color. The optimum acidity for the end-point is fixed by the addition of trisodium citrate in amount equivalent ($\frac{1}{3}$ mol.) to the free nitric acid present. Under these conditions one drop of excess $\frac{N}{50}$ iodide gives a color perceptible in 150 cc. of solution.

The following solutions are required:

I. An acid $M/29.25$ solution of silver nitrate, 1 cc. of which is equivalent to 2 mg. of $NaCl$.⁵

$AgNO_3$	5.812 gm.
HNO_3 (sp.gr. 1.42)	250 cc.
Water to	1000 cc.

In place of silver nitrate, metallic silver, which can be obtained in a high state of purity, may be made the basis of the solution. In this case 3.688 grams of silver are dissolved in the nitric acid and diluted to 1000 cc. We also find it convenient to prepare a stock solution of silver nitrate ten-fold as concentrated as the above, and prepare the $M/29.25$ solutions from this by dilution. As a check on its accuracy the stock solution may be standardized against a known hydrochloric acid solution by the Volhard method, or gravimetrically.

II. A solution of $M/58.5$ potassium iodide, 1 cc. of which is equivalent to 1 mg. of $NaCl$.

KI	3.0 gm.
Water to	1000 cc.

This solution is standardized against the silver solution by adding 5 cc. of the latter to 5 cc. of Solution III and titrating with the iodide solution to the blue end-point. The iodide solution is then diluted to such a degree that 10 cc. are exactly equivalent to 5 cc. of the silver solution.

⁵ The total chlorides are, of course, determined by the method. For the sake of convenience, all estimations here reported are calculated as sodium chloride.

III. A solution, for use in the final titration, containing sodium citrate, sodium nitrite, and starch, which substances respectively regulate the acidity, provide an oxidizing agent for the iodide, and serve as indicator.

Sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 + 5\frac{1}{2}\text{H}_2\text{O}$)	446	gm.
Sodium nitrite	20	gm.
Soluble starch	2.5	gm.
Water to	1000	cc.

The starch is first dissolved with the aid of heat in about 500 cc. of water. The citrate and nitrite are then added, and the mixture is heated until all is dissolved. The solution, while still hot, is filtered through cotton, the filter washed with hot water, the filtrate allowed to cool, and made up to 1000 cc. Filtration removes insoluble substances occurring chiefly in the nitrite, and cotton filters more rapidly than filter paper. The solution keeps indefinitely. It becomes cloudy on standing, but its efficacy is not impaired.

In detail, the titration of the protein-free plasma filtrate is carried out as follows: Either 10 cc. of the filtrate, containing the chlorides of 1 cc. of plasma, are taken in a pipette for titration, or the filtrate is collected directly in a certified 25 cc. graduated cylinder, where it is measured, so that the entire amount may be taken for titration. In this way 13 to 14 cc. of filtrate, corresponding to 1.3 to 1.4 cc. of plasma, may be obtained for titration. In this case the cylinders must be accurate to within 0.05 cc. Ordinary 25 cc. cylinders are not to be used, as errors in graduation up to 0.2 cc. or more are quite common, and unless certified cylinders are available the use of a 10 cc. pipette is advisable.

After either measuring 10 cc. of the filtrate into a 25 cc. volumetric flask or recording the amount of filtrate obtained in the cylinder, 5 cc. of the acidified $M/29.25$ silver nitrate solution (Solution I) are added, and the whole is made to the 25 cc. mark with water. This will precipitate up to 10 mg. of NaCl. In samples with high percentage of chloride, only enough filtrate is taken to keep within this limit of 10 mg. Two drops of octyl (caprylic) alcohol are added, and the vessel is stoppered and shaken gently by inverting it several times. Immediate coagulation of the silver chloride occurs. After allowing five minutes for complete precipitation to occur, the

solution is filtered through a dry folded filter, and a perfectly clear and colorless filtrate again obtained. In extracts from whole blood the silver chloride does not coagulate so readily as in plasma extracts, but it yields an equally clear filtrate. In any case some silver chloride may pass through the filter with the first few drops, but it is necessary only to pass them through the same filter again to obtain a filtrate perfectly clear to the eye. Observed in the nephelometer the turbidity of these filtrates corresponds to only 0.01 mg. or less of sodium chloride.

An aliquot part of the filtrate—20 cc.—is now taken with a pipette for titration. Or, if so desired, the precipitate and filter may be washed several times with water and the entire filtrate titrated. The use of an aliquot part, however, saves time and appears to be equally accurate. Just before titration with the potassium iodide solution (Solution II) one adds a volume of the citrate solution (Solution III) equal to the volume of Solution I represented in the filtrate to be titrated. If, as is usually the case, one has used 5 cc. of Solution I diluted to 25 cc., and taken 20 cc. of the filtrate for titration, one has the equivalent of 4 cc. of Solution I present, and must accordingly add 4 cc. of Solution III. On adding Solution III a slight turbidity appears, which in no way interferes with the end-point.

The potassium iodide solution is then run in from a burette until the blue end-point appears. The first definite blue color is taken as the end-point, and with a slight practice is unmistakable. An additional drop, which may be used as a control, causes such a deep blue color that it is impossible to make an error of more than one drop in titration. Should the end-point be accidentally passed, one may add 1 cc. of Solution I, 1 cc. of Solution III and retitrate, allowing in the calculation for the extra silver nitrate.

The result may be calculated from the following formula, which applies only when 20 cc. of the filtrate from silver chloride are titrated:

$$\text{Grams NaCl per liter} = \frac{12.5 (8 - \text{cc. KI solution used})}{\text{cc. blood filtrate taken.}}$$

Thus, if 13 cc. of filtrate are obtained and titrated after precipitation of plasma protein, and 1.60 cc. of KI are used in the final titration,

$$\text{Grams NaCl per liter} = \frac{12.5 (8 - 1.60)}{13} = 6.15.$$

In case only 1 cc. of plasma instead of 2 cc. has been used, the factor 25 replaces 12.5 in the numerator. In case the entire filtrate from the silver chloride instead of 0.8 of it (20 cc.) is taken for the final titration, the formula is $\frac{10 (10 - \text{cc. KI solution})}{\text{cc. blood filtrate}}$ when 2 cc. of plasma are used, or $\frac{20 (10 - \text{cc. KI solution})}{\text{cc. blood filtrate}}$ when only 1 cc. is taken.

Accuracy of the Method.

The fact that the titration method for chlorides is capable of an accuracy of one part per one thousand has been previously established.⁶ The exactness of the above application of the method depends directly on the care with which measurements are made and on the purity of the reagents used. All reagents must, of course, be free from substances precipitated by silver, and are easily tested. As the volumes measured are not large, however, small absolute errors in measurement may cause considerable percentage error in the final result. All glassware must therefore be carefully calibrated by either the weight of water contained (flasks, cylinders, 2 cc. pipettes) or the weight delivered (other pipettes, burettes), and burettes should be used which are capable of being read to 0.02 cc. and which deliver small drops. If these precautions are followed, one should uniformly obtain results as shown in Table I, which are well within a limit of error of 1 per cent.

Control Determinations by Fusion Method.

As stated above, the accuracy of the titration method itself has been established to a degree well beyond the present requirements of blood analysis. In the present application of the method, however, additional manipulation (removal of colloids by coagulation and charcoal) has been introduced in order to eliminate interfering substances, particularly proteins, before precipitating the chlorides. In order to show both that this preliminary manipulation introduces no error, and that it is efficient in removing interfering substances,

⁶ McLean and Van Slyke: *loc. cit.*

we have compared the results obtained after coagulation with those after fusion, by which all the organic material of the plasma was destroyed. The fusions were done with sodium carbonate and lime, according to the method described by H. Schiff,⁷ which accomplishes destruction of the organic matter without the formation

TABLE I.

Comparison of Results Obtained by Coagulation and Fusion Methods. In Each Case 5 Cc. of M/29.25 AgNO₃ Solution Were Used, the Volume Was Made to 25 Cc., and 20 Cc. of Filtrate Were Titrated with M/58.5 KI Solution.

COAGULATION					FUSION, 1 CC. SAMPLE	
Sample	Dilution	Filtrate titrated	M/58.5 KI used	NaCl per cc. of sample	M/58.5 KI used	NaCl per cc. of sample
Normal plasma* I.	20 : 200	cc.	cc.	mg.	cc.	mg.
" " "		12.5	1.95	6.04	3.15	6.06
" " "		12.5	1.97	6.03	3.18	6.02
" " "		12.5	1.96	6.03		
" " "		12.5	1.98	6.01		
" " "		12.5	2.00	6.00		
" " II.	2 : 20	13.20	1.72	5.95	3.23	5.96
" " "	2 : 20	13.60	1.50	5.97		
" " III.	2 : 20	13.10	1.73	5.98	3.22	5.98
" " "	2 : 20	12.20	2.12	6.02		
" " IV.	2 : 20	14.60	0.82	6.14	3.07	6.16
" " "	2 : 20	13.60	1.35	6.11		
Nephritic plasma I.	2 : 20	13.80	0.80	6.52	2.75	6.56
" " II.	2 : 20	13.60	1.24	6.21	3.05	6.19
Pneumonia plasma.	2 : 20	11.50	2.40	5.29	4.20	5.25
Diabetic plasma.	2 : 20	14.00	2.35	5.04	4.0	5.00
Whole blood.	2 : 20	9.90	4.12	4.90	4.05	4.94
Ascitic† fluid.	2 : 20	14.80	0.42	6.39	2.92	6.35

* Determination by Volhard method on 15 cc. sample gave 6.06 mg. NaCl per cc. of plasma.

† Determination by Volhard method on 15 cc. sample gave 6.35 mg. NaCl per cc. of fluid.

of cyanides. One cc. of plasma was mixed in a platinum crucible with 1 gram of a mixture composed of 1 part of Na₂CO₃ to 4 of CaO, dried on an electric hot plate, covered with 0.5 gram of the same fusion mixture, burned for five minutes over a Chaddock burner, and the mixture dissolved in an excess of nitric acid. The solution was made neutral with 60 per cent NaOH, concentrated on the steam bath to about 10 cc., transferred to a 25 cc. volumetric flask,

⁷ H. Schiff: *A. Classens Ausgewählte Methoden der analytischen Chemie*, Braunschweig, 1903, ii, p. 763.

TABLE II.

Results of Duplicate Analyses of Plasma by Coagulation Method. Dilution in Each Case 1: 10, 5 Cc. of AgNO₃ Solution Were Used, the Volume Was Made to 25 Cc., and 20 Cc. of Filtrate Were Titrated with KI.

SAMPLE	FILTRATE TITRATED	M/58.5 KI USED	NaCl PER CC. OF PLASMA	DIFFERENCE
	cc.	cc.	mg.	
1.	13.90	1.40	5.94	
	12.35	2.10	5.97	0.03
2.	13.05	1.72	6.01	
	13.15	1.65	6.04	0.03
3.	14.15	1.63	5.63	
	13.20	2.05	5.64	0.01
4.	13.30	1.54	6.08	
	13.85	1.30	6.05	0.03
5.	13.10	1.60	6.11	
	13.45	1.45	6.09	0.02
6.	13.80	1.50	5.89	
	13.35	1.72	5.88	0.01
7.	14.15	1.22	5.99	
	13.0	1.77	5.99	0.00
8.	13.20	1.72	5.95	
	13.60	1.50	5.97	0.01
9.	12.60	1.92	6.19	
	13.1	1.50	6.19	0.00
10.	13.20	1.72	5.95	
	13.60	1.50	5.91	0.04
11.	12.5	1.95	6.04	
	12.5	1.97	6.03	
	12.5	1.96	6.03	
	12.5	1.98	6.01	
	12.5	2.00	6.00	0.04
12.	13.1	1.73	5.98	
	12.2	2.12	6.02	0.04
13.	14.6	0.82	6.14	
	13.6	1.35	6.11	0.03
14.	12.75	1.75	6.13	
	13.6	1.35	6.11	0.02
15.	13.15	1.70	5.99	
	12.1	2.18	6.01	0.02
16.	12.4	1.81	6.23	
	12.10	1.96	6.24	0.01
17.	13.90	1.16	6.15	
	13.40	1.45	6.11	0.04
18.	12.85	1.84	5.99	
	12.55	2.0	5.98	0.01
19.	12.55	2.0	5.98	
	14.45	1.04	6.02	0.04

and cooled. 5 cc. of the M/29.25 silver nitrate solution were added, the whole was made to volume, shaken with octyl alcohol, filtered, and 20 cc. of filtrate were titrated as above. Results by this method correspond, as is shown in Table I, very closely to those obtained when proteins are removed by coagulation.

Table II shows results of duplicate analyses by the coagulation method on the same sample. Duplicates agree almost invariably within 0.05 mg. of each other, and usually closer. It is probable that the average error in determining the chlorides in 2 cc. of plasma is much less than 1 per cent.

Application to Urine.

The procedure is not superior to the Volhard for ordinary urine titrations. Results fully as accurate as those by the Volhard, however, can be obtained with much less material, and this may make the new method valuable for certain work, such as in comparing the chloride contents of urines collected separately from both ureters, or titrating urines of small animals.

TABLE III.

Albumin-Free Urines, Not Treated with Heat and Acetic Acid, Dilution 1: 50, Including 10 Cc. of M/29.25 AgNO₃ Solution in Each Case. 25 Cc. of Filtrate Were Titrated with KI.

SAMPLE	M/58.5 KI USED	NaCl PER CC. OF URINE	VOLHARD NaCl PER CC. OF URINE
	cc.	mg.	mg.
Normal urine	3.7	12.6	12.6
Normal urine	3.45	13.1	13.0
Normal urine	4.48	11.04	11.1
Normal urine	5.05	9.9	9.8
Normal urine	7.7	4.6	4.6
Normal urine	5.8	8.4	8.3
Normal urine	5.3	9.4	9.4
	3.35	13.0	13.0
	4.28	11.44	11.5

TABLE IV.

Albuminous Urines Treated with Magnesium Sulphate, Acetic Acid, and Heat. 5 Cc. of AgNO₃ Solution Were Used in Each Case, the Solution Was Made to 25 Cc., Filtered, and 20 Cc. of Filtrate Were Taken for Titration.

SAMPLE	DILUTION	FILTRATE TITRATED	M/58.5 KI USED	NaCl PER CC. OF URINE	VOLHARD NaCl PER CC. OF URINE
		cc.	cc.	mg.	mg.
Nephritic urine + albumin 6.0% . . .	1 : 20	10.00	4.74	8.16	8.15
Nephritic urine + albumin 5.7% . . .	2 : 20	12.50	5.2	2.80	2.70
Diabetic urine + albumin trace . . .	1 : 20	10.00	5.85	5.38	5.30

When the urine contains no albumin, it is necessary only to measure 1 cc. into a 50 cc. volumetric flask half filled with water, add 10 cc. of the silver nitrate solution, fill to the mark with water, add 2 to 3 drops of octyl alcohol, shake, and filter. Twenty-five cc. of the filtrate are taken with a pipette, 5 cc. of citrate solution added, and the excess silver is titrated with iodide. 2 (10—cc. KI) then gives mg. NaCl in 1 cc. of urine, or grams NaCl per liter, and the method as thus applied has a range of 0 to 20 grams per liter. In the case of urines containing albumin, the procedure is the same as for blood, 2 cc. being diluted to 20 cc. with magnesium sulphate, acetic acid, and water, and coagulated. Ten cc. of the filtrate, representing 1 cc. of urine, are then treated as above described.

Tables III and IV give a comparison of results with urines analyzed by the present method and the Volhard titration, respectively, the titrations with the latter being performed in the presence of the silver chloride precipitate.

Other Applications.

Whole blood, serum, and ascitic, or pleural fluid are treated in the same way as plasma. For the determination of chlorides in gastric juice, etc., the same manipulation may be carried out, the volume of filtrate used for titration depending on the chloride content.

THE OCCURRENCE OF CARRIERS OF DISEASE-PRODUCING TYPES OF PNEUMOCOCCUS.

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In previous papers¹ we have discussed the varieties of pneumococcus responsible for the production of lobar pneumonia and the differences that exist between such organisms and those found in the mouths of healthy individuals. The pneumococci obtained from persons suffering from lobar pneumonia have been divided into four groups. We have pointed out that the first three groups are comprised of disease-producing races, and are responsible for about 75 per cent of all cases of lobar pneumonia. Approximately 25 per cent of cases of pneumonia are due to the fourth group. The pneumococci of this group cannot readily be distinguished from those dwelling in the normal human mouth. Disease caused by the highly parasitic types is usually much more severe than that occasioned by the strains which are indistinguishable from the sputum pneumococci. Inasmuch as the highly virulent forms are always associated with disease, and only occur in the mouths of healthy individuals under the special conditions which are reported in this paper, the evidence is strong that in the spread of lobar pneumonia the disease in a majority of instances is transmitted from one individual to another. Infectious diseases usually spread by immediate contact, through the intermediation of a temporary host, or by the agency of a healthy carrier. The importance of these modes of transmission varies with different diseases and in many instances more than one of these mechanisms may be involved. In all likelihood the healthy carrier may be a greater menace to the health of the community than the

¹ Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.*, 1913, lxi, 727. Dochez, A. R., and Avery, O. T., *Jour. Exper. Med.*, 1915, xxi, 114.

infected individual, largely because of failure to recognize the carrier condition. In the epidemiology of certain diseases, notably typhoid and epidemic cerebrospinal meningitis, the importance of the carrier state is well known because of the definite tracing of foci of disease to such a condition. The more readily a disease is transmitted from one individual to another, the greater becomes the number of instances at any one time, and consequently the more vigorous the search for the source of infection. The sporadic occurrence of lobar pneumonia, combined with the inability to distinguish disease-producing types of pneumococcus from those habitually living in normal mouths, has probably been responsible for the failure to establish a well defined epidemiology for this disease.

It is not our purpose in this paper to discuss the importance of immediate contact as a cause of the spread of pneumonia, for, although instances have occurred in which two closely associated individuals, such as husband and wife, have been infected within a short period with the same type of pneumococcus, cases of pneumonia usually develop at such wide intervals of space and time that direct contact relationships are obscured. Inasmuch as fully 75 per cent of all cases of lobar pneumonia are caused by peculiarly distinct races of pneumococci, not occurring in the normal mouth, then, as we have previously assumed, these instances of the disease must be due to contact infection, either direct or indirect, and some mechanism must exist by means of which the etiological agent is transmitted from one individual to another. In the present study we shall show that persons closely associated with individuals suffering from pneumonia in a large percentage of instances harbor in their mouths pneumococci of the same type as those causing the disease, that such organisms are not found in the mouths of normal individuals not exposed to pneumonia, and that the considerable period of time during which these organisms are carried may in part account for the sporadic occurrence of individual cases of pneumonia. An additional means of transmission exists in the fact that the recovered case also may carry the pneumococcus responsible for his disease during a relatively long period of time.

In the present paper are presented the studies of the pneumococci obtained from the mouths of persons associated with cases of pneumonia, of the type of pneumococcus encountered in the mouths of normal unexposed individuals, and of the period of time during which convalescents harbor the disease-producing organisms. For

TABLE I.

Incidence of Carrier Condition in Healthy Individuals in Contact with Lobar Pneumonia.

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Type found in associates.	Duration of period of carrying.
2276	Type I	Wife	Type I	10 days +
2195	Type I	Husband	Type I	24 days.
2203	Type I	Wife	Type IV	
		Mother	Type IV	
2237	Type I	Nurse	Type I	Undetermined.
W	Type I	Wife	Type IV	
B	Type I	Wife	No pneumococcus	
		Daughter	Type IV	
2269	Type I	Wife	Type IV	
2286	Type II	Mother	Type IV	
		Father	Type IV	
2299	Type II	Sister	No pneumococcus	
2301	Type II	Wife	No pneumococcus	
2294	Type II	Wife	No pneumococcus	
2309	Type II	Mother	Type IV	
2314	Type II	Wife	Type II	Undetermined.
2335	Type II	Mother	No pneumococcus	
		Sister	No pneumococcus	
2326	Type II	Wife	No pneumococcus	
2330	Type II	Daughter	Type II	Undetermined.
2344	Type II	Sister	No pneumococcus	
1835	Type II	Wife	Type II	9 days +
2174	Type II	Son	Type IV	
2175	Type II	Mother	Type II	27 days +
		Sister	No pneumococcus	
		Nurse	Type II	Developed pneumonia, Type II.
2202	Type II	Mother	Type II	45 days.
2199	Type II	Husband	Type IV	
2298	Type II	Sister	Type IV	
2226	Type II	Wife	Type IV	
2245	Type II	Mother	Type IV	
		Brother	Type II	39 days +
2230	Type II	Wife	No pneumococcus	
2247	Type II	Daughter	Type II	Undetermined.
		Wife	No pneumococcus	
		Son	Type IV	
2266	Type II	Wife	No pneumococcus	
		Brother-in-law	No pneumococcus	
2270	Type II	Mother	Type II	7 days +
		Sister	No pneumococcus	
2265	Type II	Physician	Type II	21 days.

TABLE I.—*Concluded.**Summary.*

Type of pneumococcus.	No. of cases.	Positive contacts.	Per cent.
Type I.....	8	3	37.5
Type II.....	24	10	41.7
Total.....	32	13	40.6

Approximate duration of carrier state 23 days.

the sake of clearness, an explanation of our classification of pneumococci is given. The organisms have been placed into four groups, numbered from I to IV. Groups I, II, and III are found only in association with disease and are distinctly parasitic in type. Members of Groups I, II, and III are recognized by their immune reactions which are identical within the respective group. Group IV consists of a heterogeneous series of strains which are not related antigenically, and which cause a minority of cases of pneumonia, and from which the pneumococcus occurring in the normal mouth is indistinguishable. In the Tables I to IV the pneumococci studied are classified according to this numerical grouping.

TABLE II.

Type of Pneumococcus Isolated from Sputum of Normal Individuals.

Pneumococcus.	Incidence.	Per cent.
Contacts { Type I	3	2.6
Type II	8	7.0
Type IV	55	48.6
No pneumococcus	47	41.6
Total	113	
Pneumococcus present	66	58.4
Pneumococcus absent	47	41.6

TABLE III.

Type of Pneumococcus Isolated from Individuals with Lobar Pneumonia.

Pneumococcus.	No. of cases.	Per cent.
Type I	78	34.97
Type II	75	33.63
Type III	22	9.86
Type IV	48	21.52
Total No. of cases	223	

TABLE IV.

Persistence of Disease-Producing Type of Pneumococcus during Convalescence.

Case No.	Type of pneumococcus during height of disease.	Type of pneumococcus after recovery.
1654	Type I	60 days, Type I. 65 days, Type IV.
1751	Type I	59 days, streptococcus. 73 days, streptococcus.
1775	Type I	30 days, Type IV.
1867	Type I	90 days, Type I.
1828	Type I	33 days, Type IV.
1792	Type I	30 days, Type IV.
2167	Type I	12 days, Type IV.
2168	Type I	45 days, Type I.
2191	Type I	15 days, Type IV.
2195	Type I	28 days, Type I. 49 days, no pneumococcus.
2203	Type I	12 days, no pneumococcus.
2237	Type I	13 days, Type I.
W	Type I	15 days, Type I. 23 days, Type IV.
B	Type I	14 days, no pneumococcus.
2250	Type I	10 days, Type I.
2269	Type I	13 days, no pneumococcus.
2284	Type I	15 days, Type I. 29 days, no pneumococcus.
2267	Type I	15 days, no pneumococcus.
2276	Type I	15 days, Type I.
1679	Type II	30 days, streptococcus. 48 days, Type IV. 108 days, Type IV.
1753	Type II	60 days, Type IV.
1763	Type II	34 days, streptococcus. 40 days, Type IV.
1761	Type II	47 days, streptococcus. 78 days, Type IV.
1825	Type II	53 days, Type II.
1786	Type II	20 days, Type IV. 25 days, Type IV.
1820	Type II	30 days, Type II.
1827	Type II	14 days, Type IV.
1880	Type II	63 days, Type II.
1950	Type II	21 days, Type IV.
1969	Type II	24 days, Type IV.
2174	Type II	15 days, streptococcus.
2175	Type II	17 days, no pneumococcus.
2212	Type II	12 days, Type II. 17 days, no pneumococcus.

TABLE IV.—*Concluded.*

Case No.	Type of pneumococcus during height of disease.	Type of pneumococcus after recovery.
2202	Type II	37 days, Type II. 43 days, Type IV.
2199	Type II	32 days, no pneumococcus.
2226	Type II	29 days, Type II.
2245	Type II	16 days, Type IV.
2266	Type II	15 days, Type II.
2270	Type II	37 days, Type II.
2286	Type II	10 days, Type II. 25 days, no pneumococcus.
2292	Type II	29 days, Type IV.
2296	Type II	19 days, no pneumococcus.
1743	Type III	13 days, Type III. 73 days, Type IV.
2185	Type III	16 days, Type III. 34 days, no pneumococcus.
2249	Type III	14 days, Type III.

In the above four tables are given the main facts upon which we base our assumption that in the majority of cases lobar pneumonia is a disease the continued wide-spread incidence of which is dependent upon communication of infection from one individual to another. Table I establishes beyond doubt the existence of healthy carriers of the disease-producing types of pneumococcus. The study of the carrier state was limited to the investigation of infection with pneumococcus Types I and II because of the relative ease with which these organisms can be distinguished from other types of pneumococcus. Out of a total of thirty-two cases studied, at least one carrier of infection among the patient's associates was found in thirteen instances, 40.6 per cent. Types I and II show approximately the same percentage incidence of the carrier condition, and in every instance the pneumococcus isolated corresponds in type with that of the infected individual. The approximate duration of the carrier state has been twenty-three days, which is probably somewhat shorter than would be found had it been possible to retain under observation all carriers until the disease-producing type of pneumococcus had disappeared from the mouth

flora. Study of Table I shows that positive carriers are more commonly observed among females than among males, a fact that is probably accounted for by the more frequent service of the former in a nursing capacity, thus entailing more intimate association with the sick.

Tables II, III, and IV develop somewhat further, points brought out in a previous communication. In Table II is shown the incidence of pneumococcus in the mouth flora of normal individuals and the classification of such pneumococci. All normal individuals studied are given in this table, whether in contact with cases of lobar pneumonia or not. Pneumococcus was found in 58.4 per cent of all instances and was absent in 41.6 per cent. Of the cases in which pneumococcus has been found, 48.6 per cent of the organisms have been of the sputum type and 9.6 per cent have been of the disease-producing type. All the latter have occurred in individuals intimately associated with cases of lobar pneumonia, and their presence is dependent upon this association. Determination of contact carriers of pneumococci belonging to Group III, *Pneumococcus mucosus*, presents certain difficulties. Only recently have we been able to obtain an immune serum effective against organisms of this group. Further development of the study of the mucous group is showing that certain organisms resembling in their cultural reactions *Pneumococcus mucosus*, are, in reality, mucous types of streptococcus. The latter varieties are frequently found in normal sputum, and have led to some confusion. With the working out of the serological reactions of the true *Pneumococcus mucosus*, evidence is accumulating that this organism is as strictly pathogenic in type, and has quite as specific immunological characteristics as pneumococci belonging to Groups I and II.

For comparison with the statistics given in Table II, which illustrates the prevalence of the mouth type of pneumococcus in normal individuals, Table III is added to show the percentage incidence of the fixed types of pneumococci observed in individuals suffering from lobar pneumonia. In this table the highly pathogenic types are dominant, being responsible for 77.2 per cent of all infections studied. This fact is convincing evidence that specific types of

pneumococci are mainly responsible for the production of lobar pneumonia. The dominance of these organisms in disease is in striking contrast to the high percentage incidence of the sputum type of pneumococcus in the mouth flora of normal individuals.

Table IV shows the length of time during which recovered cases of pneumonia harbor the organism responsible for their disease. The period of carrying is measured from the date of onset of the pneumonia. The shortest time in which the disease-producing pneumococcus has disappeared from the mouth has been twelve days, and the longest duration of carrying has been ninety days. These results show that pathogenic types of pneumococcus persist in the mouths of individuals recovering from lobar pneumonia for a variable period of time. Because of wide intervals between observations, the average duration of this condition can only be approximately determined, and has been found to be about twenty-eight days.

The studies detailed in this paper show that there are two sources of danger in the spread of pneumococcus infection. One lies in the occurrence of healthy carriers of disease-producing pneumococci among individuals associated with cases of pneumonia, and the other in the fact that patients recovering from the disease harbor the responsible organism for a considerable length of time. The actual tracing of cases of pneumonia to examples of the carrier state is difficult, but even the small amount of effort that has so far been devoted to this side of the study has brought to light an occasional suggestive fact.

SUMMARY.

Lobar pneumonia in 75 per cent of instances is due to specific types of pneumococci possessed of a high degree of pathogenicity. Although pneumococci occur in the mouths of 60 per cent of normal individuals, such organisms are readily distinguishable from the highly parasitic types of pneumococcus responsible for the severe forms of lobar pneumonia, a convincing proof that infection in this disease is, in the majority of instances, not autogenic in nature, but is derived from some extraneous source. In a high percentage of instances healthy persons intimately associated with

cases of lobar pneumonia harbor the disease-producing types of pneumococcus. In every such instance the pneumococcus isolated has corresponded in type with that of the infected individual. Convalescents from pneumonia carry for a considerable length of time the type of pneumococcus with which they have been infected. The existence of the carrier state among healthy persons and among those recently recovered from pneumonia establishes a basis for understanding the mechanism by means of which lobar pneumonia spreads and maintains its high incidence from year to year.

THE NUMERICAL LAWS GOVERNING THE RATE OF EXCRETION OF UREA AND CHLORIDES IN MAN.¹

I. AN INDEX OF UREA EXCRETION AND THE NORMAL EXCRETION OF UREA AND CHLORIDES.

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PLATES 27 AND 28.

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That excretion of at least certain substances is carried on by the kidneys according to definite laws, capable of numerical expression, was shown first for urea by Ambard, who formulated these laws in a form known as Ambard's coefficient, and demonstrated the relative constancy of the formula (1). Similar laws were formulated by Ambard and Weill (2) for the excretion of chlorides. In a later book Ambard (3) reviews the results obtained by other workers in the field, and applies similar laws to the excretion of water and of glucose.

Ambard's work has attracted attention among French clinicians, who have used his coefficient as a means of estimating renal function. His conclusions have been criticized by writers in this country on the ground that he used the inaccurate hypobromite method for determination of urea in both blood and urine, but his critics have failed to repeat his observations with more accurate methods.

Using the methods devised in Folin's laboratory for the estimation of urea in blood and urine we have previously (4) shown the relative constancy of the Ambard formula for urea in normal individuals. We now present the results of a much larger series of

¹ Preliminary reports of the work here presented have appeared in *Am. Jour. Physiol.*, 1914-15, xxxvi, 357; *Proc. Soc. Exper. Biol. and Med.*, 1915, xii, 164; *Med. Record*, 1915, lxxxvii, 624.

observations on urea and sodium chloride excretion in individuals with normal excretion.

Ambard's Laws.

Ambard's first experiments were with dogs. He found that when the concentration of urea in the urine is constant the rate of excretion varies directly as the square of the concentration of urea in the blood. His first law thus formulated is:

$$\frac{(\text{Urea in blood})^2}{\text{Rate of excretion}} = \text{Constant, or } \frac{\text{Urea in blood}}{\sqrt{\text{Excretion per unit of time}}} = \text{Constant}$$

(when concentration in urine is constant).

Secondly, he found, by comparing different experiments in which the concentration of urea in the blood was the same, that the rate of excretion then varied inversely as the square root of the concentration in the urine; *i. e.*,

$$\frac{\text{Rate of excretion I}}{\text{Rate of excretion II}} = \frac{\sqrt{\text{Concentration II}}}{\sqrt{\text{Concentration I}}}$$

This may also be expressed

$$\text{Rate I } \sqrt{\text{Concentration I}} = \text{Rate II } \sqrt{\text{Concentration II}}$$

or, in other words, when the blood urea remains constant the rate times the square root of the concentration in the urine remains constant. Introducing this factor into the first law we have

$$\frac{\text{Urea in blood}}{\sqrt{\text{Rate of excretion}} \sqrt{\text{Concentration in urine}}} = \text{Constant.}$$

This was shown to hold true for the same animal or for the same individual, or for animals or individuals of the same or nearly the same weight. For individuals of different weight it became necessary to introduce a further modification, in order to make all individuals comparable. This modification is based on the assumption, supported by the results of experiment, that, other factors remaining constant, the rate of excretion varies directly with the weight of the individual. One assumes that the amount of active kidney tissue and the circulation of blood through the kidneys vary directly with the weight. This may be expressed as

$$\frac{\text{Rate}}{\text{Weight}} = \text{Constant (other factors remaining constant).}$$

Introducing this correction into the previous formula, we have

$$\frac{\text{Concentration of urea in blood}}{\sqrt{\frac{\text{Rate}}{\text{Weight}}} \sqrt{\text{Concentration in urine}}} = \text{Constant.}$$

This formula expresses all of Ambard's laws in the simplest form. In order to

standardize the formula for use in human subjects, it was expressed by Ambard as follows:

$$\frac{Ur}{\sqrt{D \times \frac{70}{Wt} \times \sqrt{\frac{C}{25}}}} = K \text{ (constant).}$$

Ur = gm. of urea per liter of blood.

D = gm. of urea excreted per 24 hours.

Wt = weight of individual in kilos.

C = gm. of urea per liter of urine.

This formula is known as Ambard's coefficient, and the value obtained for K by Ambard and Weill in normal human subjects ranged between 0.060 and 0.070. We have found the normal variations to be somewhat greater, and have found the usual normal to be about 0.080. The difference in the value of the constant is probably due to the more accurate method used for determination of urea. In Table I will be found the results of a larger series of observations in individuals with normal excretion.

The introduction of a standard weight of 70 kilos, a standard concentration of 25 gm. per liter, and the expression of rate of excretion as the rate per 24 hours are purely arbitrary, and can not affect the general relationship between the variable factors. When these arbitrary factors are kept constant, they, in conjunction with the constant relationship between the four variable factors, tend to keep K constant, and the actual numerical value of K is determined by these arbitrary additions to the formula. All the observations of the French school are based on the formula as thus stated.

Ambard and Weill also applied laws to the excretion of sodium chloride in human subjects. They found that the same general laws were applicable, with the important exception that, while excretion of urea occurs no matter how low its concentration in the blood falls, there is a threshold for chloride excretion, and when the concentration in the plasma falls below this threshold value, excretion of chloride practically ceases. In view of the fact that there is a wide difference in chloride content of the corpuscles and plasma, plasma alone, as the fluid part of the blood, has been studied. Ambard and Weill, partly by direct experiment and partly by plotting curves, established the normal threshold value for sodium chloride as 5.62 gm. per liter of plasma. Therefore the sodium chloride above 5.62 gm. per liter determines the rate of excretion, and the law may be expressed as for urea,

$$\frac{\text{Excess NaCl over 5.62 gm. per liter of plasma}}{\sqrt{\frac{\text{NaCl in 24 hrs.}}{\text{Wt. in kilos}}} \sqrt{\text{NaCl per liter of urine}}} = \text{Constant.}$$

For practical use it appears best to calculate the plasma sodium chloride from the rate of excretion, and to compare the calculated concentration with that actually found. The formula, as derived with the use of values actually found for the constant in the above formula, reads

$$\text{Plasma NaCl} = 5.62 + \sqrt{\frac{D \times \frac{70}{Wt} \sqrt{\frac{C}{14}}}{79.33}}.$$

(The symbols have the same meaning as in the urea formulas.)

This, in its simplest form, reads

$$\text{Plasma NaCl} = 5.62 + \sqrt{\frac{\text{Gm. NaCl per 24 hrs.} \times \sqrt{\text{Gm. NaCl per liter of urine}}}{4.23 \times \text{Wt. in kilos}}}.$$

The constancy of this formula depends on two factors: (1) the constancy of the threshold, and (2) the constancy of the rate of excretion of sodium chloride above the threshold. In their original contribution Ambard and Weill (1, 2) believed that the threshold was quite constant in normal individuals. Later they recognized some variation in the threshold in normal individuals, though this variation seems to be relatively slight. Assuming that the laws for rate of excretion of sodium chloride over the threshold remain constant in normal individuals, one may calculate the threshold by subtracting the calculated excess from the sodium chloride actually found in the plasma, and our figures for the variations in the threshold are based on the following formula:

$$\text{Threshold} = \text{Plasma NaCl} - \sqrt{\frac{D \sqrt{C}}{4.23 \text{ Wt}}}.$$

This formula is subject to error if the rate of excretion over the threshold varies. We have not found that the urea excretion gives any basis for estimating the rate of sodium chloride excretion, and accordingly have not used Ambard's combined formula for calculating the threshold of sodium chloride excretion. Our figures on the threshold are only to be regarded as approximate, since we have so far no means of recognizing variations in rate of excretion over the threshold.

The principles of the laws of urea and chloride excretion may be illustrated in a very simple way. If we imagine a vessel into which water flows at a constant rate, escaping through an outlet at the bottom, the water will seek the level in the vessel at which the pressure is such that the rate of outflow is exactly equal to the intake. If we then increase or decrease the rate of inflow, the level will change to meet the new conditions. The change in level of the fluid in the vessel may be regarded as a compensatory change. Under physiological conditions fluctuations in the level of blood urea compensate for changes in the rapidity of formation of urea, and changes in the level of chloride in the plasma compensate for fluctuations in chloride intake. Under pathological conditions changes in the level of urea and sodium chloride in the blood also occur to compensate for changes in the outlet, in the form of diseased kidneys. In the case of chlorides the outlet of the vessel must be considered as being at some distance from the bottom, and in such a case only the level of fluid above the outlet would play any part in determining the rate of outflow, which would cease when the level of fluid fell to the level of the outlet. Similarly, only the chloride above the threshold determines the rate of excretion, which practically ceases when the threshold value is reached.

An Index of Urea Excretion.

As Ambard's coefficient expresses changes in urea excretion by variations in the value of K, these variations must be expressed on

an arbitrary scale. Values for K increase directly with increase in U_r , other factors remaining the same. Changes in K then reflect changes in the blood urea, which changes occur as the square root of changes in rate of excretion. In order to express the changes in rate of excretion in a manner mathematically correct and based on a scale of 100 for the sake of comparison, we have used a formula adapted from the laws of Ambard, which we have called an index of urea excretion. An index of 100, corresponding to a value for Ambard's coefficient of 0.080, is the standard normal index, and variations are expressed directly in terms of the normal. Thus, an index of 50 indicates a rate of excretion 50 per cent of normal under the conditions of concentration in the blood and urine. The index is based on a standard normal Ambard's coefficient of 0.080. The actual normal variations in the index are shown in Table I. The derivation of the index is as follows:

$$\text{Index} = \frac{(\text{Rate of excretion found})}{(\text{Standard normal rate})} \times 100$$

(under the same conditions of weight, and concentration in blood and urine).

From the laws of Ambard:

$$1) \quad K = \frac{U_r}{\sqrt{\text{Rate}}}, \quad \therefore \text{Rate} = \left(\frac{U_r}{K} \right)^2.$$

$$\text{Similarly } 2) \quad 0.080 = \frac{U_r}{\sqrt{\text{Normal rate}}}, \quad \therefore \text{Normal rate} = \left(\frac{U_r}{0.080} \right)^2.$$

$$\text{Therefore } 3) \quad \frac{\text{Rate}}{\text{Normal rate}} = \frac{\left(\frac{U_r}{K} \right)^2}{\left(\frac{U_r}{0.080} \right)^2} = \left(\frac{0.080}{K} \right)^2.$$

$$\text{and } 4) \quad \text{Index} = 100 \times \left(\frac{0.080}{K} \right)^2 = \left(\frac{0.80}{K} \right)^2.$$

Substituting for K (Ambard's coefficient) and simplifying,

$$\text{Index} = \frac{\text{Gm. urea per 24 hrs.} \times \sqrt{\text{Gm. urea per liter of urine}} \times 8.96}{\text{Wt. in kilos} \times (\text{Gm. urea per liter of blood})^2}.$$

When $K = 0.080$, the standard normal, I (index of urea excretion) = 100.

In this form the index offers a means of measuring the rate of excretion, under the conditions found at any given time, directly in terms of the normal, and does not require the use of an empirical scale for comparison of pathological cases with the normal.

Normal Excretion of Urea and Sodium Chloride.
Methods for Observation.

Collection of Specimens.—Short periods are preferable for observation. By collecting the urine over a given period and withdrawing blood at the middle of the period, the blood sample may be assumed to represent the average for the period. If no food or water is taken during the period, and the period is not too soon after a heavy meal, the rate of excretion during the period will remain practically constant. We have taken, as a rule, a period of 72 minutes, during either the forenoon or afternoon. One-half hour before the period the subject drinks 150 to 200 cc. of water, and takes no more fluid or food until the observation period is ended. At the beginning of the period the bladder is emptied. 36 minutes later about 10 cc. of blood are taken from an arm vein into a dry tube containing about 100 mg. of powdered potassium oxalate, and mixed with the oxalate to prevent clotting. At the end of 72 minutes after the bladder was first emptied, the specimen of urine, representing the total amount secreted during the 72 minute period, is collected, carefully measured, and used for analysis. A 72 minute period is chosen since it is one-twentieth of 24 hours, and the calculation to 24 hours is made somewhat easier. It should be remembered that the expression of rate of excretion on the basis of 24 hours need bear no relation to the amount actually excreted in 24 hours. The rate is actually determined for the shorter period, and calculated to 24 hours as a standard period on which to base all results.

Methods for Analysis.—Very accurate analyses are necessary, when one desires to determine a quantitative relationship such as is here described. The urea content of whole blood² and urine are at present determined by the use of urease, the specific enzyme of soy bean adapted to quantitative determinations of urea by Marshall (5). We have used the permanent preparation of urease described by Van Slyke and Cullen, and the procedure advised by them (6). This method is capable of great refinement, and gives very accurate results when carefully controlled. Urine determinations are always

² The urea content of whole blood is slightly lower than that of plasma. Whole blood is always used.

accompanied by determinations of preformed ammonia, and the figure for urea plus ammonia is thus corrected. Duplicate analyses of blood are of advantage when a sufficient amount of blood is at hand, but with careful technique are hardly necessary. Duplicate analyses on urine are unnecessary. One must carefully control the activity of the enzyme used, by the method for standardizing it detailed by Van Slyke and Cullen; since if the activity is below the specified standard, the enzyme may fail to decompose all urea present in the time allowed. Reagents contaminated with ammonia must, of course, be avoided.

After removing the portion of whole blood for urea determination, the remainder is centrifugalized at high speed to throw down all corpuscles, and the plasma is pipetted off.⁸ The total chlorides of both plasma and urine are then determined and calculated as sodium chloride. For plasma we have used the method recently described by the author and Van Slyke (7). By this method accuracy within 1 per cent may be obtained with 2 cc. of plasma, and duplicate analyses are rarely necessary. When the plasma is allowed to stand in contact with the cells there is a slight tendency for plasma sodium chloride to diffuse into the cells. As will be seen by the following figures, this change occurs very slowly and it is necessary only to centrifugalize within two to three hours to avoid this danger. We have usually centrifugalized within an hour after the blood was drawn.

Effect of the Time of Separation of Plasma from Cells on the Chloride Content of Plasma.

	Plasma separated from red cells by centrifuge.		
	At once, NaCl per liter.	After	NaCl per liter.
	gm.	hrs.	gm.
Sample I.	6.11	3	6.09
" II.	5.95	3	5.97
" III.	5.99	4	5.99
" IV.	5.89	4	5.88
" V.	6.13	24	6.07

Urine chlorides are determined by a modified Volhard titration. Titration of the excess silver with ammonium sulphocyanate is per-

⁸ Serum of clotted blood is never used.

formed in the presence of the silver chloride precipitate and of 5 per cent nitric acid, after five minutes have been allowed for complete precipitation of the chloride. With this method removal of the proteins is unnecessary, as has been shown by Bayne-Jones (8), and results are uniformly accurate to within 0.2 of a gram per liter, which is sufficient for the present purposes.

From the urea and chloride determinations the values obtained are substituted in the proper formulas as described.

The administration of water before the period is in order to prevent apparent retention due to dehydration of the organism. If a fair amount of urine is thus obtained one need never obtain results in normal individuals simulating those in subjects actually retaining urea. Apparently sodium chloride is still less dependent on water intake than urea. Diet, especially as regards chloride and nitrogen intake, is unimportant from the standpoint of the observations, as the formulas are independent of the intake. It is therefore unnecessary to put an individual on a standard weighed diet in order to obtain comparable observations.

Calculation.—The substitution of values found by analysis in the formulas and the calculation of the formulas is in itself a considerable task if the ordinary arithmetical processes are used. Logarithms are of advantage, but they are also laborious. To simplify the process of calculation, and thereby reduce both the labor and the chances of error, a slide-rule has been adapted to the formulas. By the use of this device it is not even necessary to remember the formulas; the whole calculation becomes a matter of only a few seconds, and is purely mechanical. Figs. 1 and 2 show the rule as adapted to the calculation of both urea and sodium chloride formulas. It is the usual form of ten inch slide-rule, with the addition of certain scales and indices. The manipulation is quite simple and rapid, requiring no knowledge of the mathematical principles involved in the formulas.⁴

⁴ The rule, with directions for use, may be obtained from Keuffel and Esser Co., 127 Fulton Street, New York.

The same rule is adaptable to all problems of multiplication and division, and is of great service in all laboratory calculations.

The Excretion of Urea.

Table I includes all observations made on urea excretion in normal individuals, including those previously reported by McLean and Selling (4). This table also includes observations made in a few hospital patients with normal excretion and selected for this purpose. It does not include a much larger series of observations made in hospital patients in whom the urea figures fell within normal limits, but who could not be properly classified as patients with normal kidneys. The patients utilized were, for the most part, cases with heart lesions, fully compensated, without signs of renal involvement, and, except where specified, not under the influence of drugs. These cases, in conjunction with those strictly normal, furnish a basis of comparison for study of patients with abnormal excretion.

Discussion of Table I.—As will be seen, we have calculated in each case, in addition to the index of urea excretion, the two formulas

$$\sqrt{\frac{D}{W_t}} \sqrt{C} \quad \text{and} \quad \frac{U_r}{\sqrt{\frac{D}{W_t}} \sqrt{C}}.$$

As stated above, the latter formula expresses the laws of Ambard in the simplest form, without distortion by addition of the constants used in Ambard's coefficient; and we have used this formula as a test of the validity of the laws and the constancy of the normal relationship. A great part of the apparently considerable variation in the index is due to the construction of the formula, in that the index varies directly with one factor in the law; *i. e.*, with the rate of excretion. To show the actual deviation from the law we have adopted 0.30 as a normal value for

$$\frac{U_r}{\sqrt{\frac{D}{W_t}} \sqrt{C}}$$

corresponding to an index of 100, and have tabulated the percentage variation from the law as expressed in this way. Deviations where the rate of excretion is greater than that corresponding to the standard value of 0.30 are indicated by +, those less by —.

TABLE I.*

The Relation of the Rate of Urea Excretion to Concentration in Blood in 107 Observations in Individuals with Normal Excretion, Arranged according to Concentration of Urea in the Blood.

Index of urea excretion calculated from the formula,

$$\text{Index (I)} = \frac{\text{Gm. per 24 hrs. } \sqrt{\text{Gm. per liter}} \times 8.96}{\text{Wt. in kilos} \times (\text{Blood urea})^2}$$

No.	Subject.	Date.	Weight.	Urine per 24 hrs.	Urea.				$\sqrt{\frac{D}{Wt.}}$	$\sqrt{\frac{D}{Wt.} \times \sqrt{C}}$	Deviation from standard normal.	Remarks.
					Gm. per liter of blood Ur.	Gm. per liter of urine C.	Gm. per 24 hrs. D.	Index of excretion I.				
			kilos	cc.							per cent	
1	V.S....	May 5	72.0	2,300	0.191	7.85	18.0	170	0.84	0.23	+23	
2	F.C.M....	" 5	80.0	1,760	0.206	13.1	23.0	220	1.02	0.21	+30	
3	F.C.M....	" 5	80.0	2,560	0.206	9.5	24.3	196	0.97	0.21	+30	
4	A.R.D....	" 5	67.0	1,560	0.206	10.6	16.6	170	0.90	0.23	+23	
5	F.C.M....	" 17	81.6	2,800	0.211	10.1	28.2	220	1.05	0.20	+33	
6	E.S....	" 19	86.0	1,125	0.212	19.75	22.2	228	1.07	0.20	+33	
7	F.C.M....	Apr. 7	77.2	860	0.214	21.0	18.0	108	1.03	0.21	+30	
8	E.S....	May 19	86.0	1,295	0.220	14.4	18.7	153	0.91	0.24	+20	
9	F.C.M....	Jan. 20	80.0	1,073	0.221	18.14	19.45	190	1.02	0.22	+27	
10	V.S....	May 5	72.0	5,100	0.224	3.82	19.5	95	0.73	0.31	- 3	
11	D.A.P....	Apr. 8	60.0	1,900	0.225	8.35	15.9	136	0.88	0.25	+16	
12	V.S....	May 18	72.0	736	0.228	16.8	12.4	122	0.84	0.27	+10	
13	V.S....	" 18	72.0	600	0.232	13.62	8.17	70	0.65	0.36	-10	
14	2,313....	Jan. 22	67.0	1,150	0.232	11.5	13.2	110	0.82	0.28	+ 7	Neurasthenia.
15	R.A.S....	Apr. 10	70.0	1,150	0.235	21.0	24.2	255	1.26	0.19	+37	
16	F.C.M....	May 4	80.0	3,400	0.254	9.2	31.2	164	1.09	0.23	+23	
17	F.C.M....	" 6	77.2	4,248	0.256	8.0	31.2	157	1.07	0.24	+20	
18	A.M.C....	" 19	68.2	1,125	0.261	11.1	28.7	175	1.18	0.22	+27	
19	2,333....	Apr. 5	72.2	1,120	0.266	16.4	18.4	130	1.01	0.26	+13	Aortic regurgitation.
20	H.T.C....	May 19	65.0	1,282	0.266	17.94	23.0	190	1.21	0.22	+27	
21	2,333....	" 1	68.4	920	0.276	14.9	13.7	91	0.88	0.31	- 3	
22	A.M.C....	May 19	68.2	4,850	0.280	6.15	29.8	123	1.04	0.26	+13	
23	2,316....	Feb. 16	73.2	1,000	0.286	16.42	16.42	100	0.95	0.30	0	Aortic regurgitation. Heart block.
24	2,321....	Apr. 5	60.2	3,840	0.286	7.2	27.7	134	1.11	0.26	+13	
25	2,333....	" 9	73.0	4,800	0.286	7.85	37.6	159	1.20	0.24	+20	
26	F.C.M....	Mar. 18	80.0	1,380	0.286	19.15	26.5	159	1.20	0.24	+20	
27	2,321....	Apr. 1	60.0	1,380	0.296	15.1	20.8	138	1.16	0.26	+13	Heart block. Aortic regurgitation.
28	2,365....	May 5	59.8	1,900	0.296	10.9	20.6	116	1.07	0.28	+ 7	
29	H.K.A....	Apr. 8	60.0	700	0.298	21.0	14.7	114	1.06	0.28	+ 7	

* Those with initials italicized were healthy normal adults. The numbers are hospital records.

TABLE I.—Continued.

No.	Subject.	Date.	Weight.	Urine per 24 hrs.	Urea.				$\sqrt{\frac{D}{Wt} \times \sqrt{C}}$	Ur	$\sqrt{\frac{D}{Wt} \times \sqrt{C}}$	Deviation from standard, normal.	Remarks.
					Gm. per liter of blood Ur.	Gm. per liter of urine C.	Gm. per 24 hrs. D.	Index of excretion I.					
			<i>kilos</i>	<i>cc.</i>								<i>per cent</i>	
30	F.C.M...	Oct. 6	78.0	1,120	0.298	22.3	25.0	153	1.2	0.24	+20		
31	F.C.M...	June 1	77.2	720	0.299	20.7	14.7	87	0.93	0.32	-7		
32	2,365....	May 4	59.8	1,460	0.301	12.1	17.7	102	1.02	0.30	0		
33	2,365....	" 27	65.2	1,940	0.304	14.3	27.7	155	1.26	0.24	+20		
34	A.T.D...	" 29	80.0	1,580	0.305	19.5	30.8	164	1.38	0.24	+20		
35	2,313....	Feb. 18	66.2	2,050	0.309	9.85	20.2	89	0.98	0.31	-3		
36	2,333....	Apr. 30	72.2	1,860	0.316	18.0	33.5	176	1.40	0.23	+23		
37	E.S....	Mar. 19	90.0	730	0.316	28.0	20.5	110	1.10	0.129	+3		
38	2,247....	Dec. 21	62.2	1,343	0.316	13.25	17.8	93	1.02	0.31	-3		Multiple sclerosis. Chronic arthritis.
39	2,183....	Nov. 17	67.1	1,690	0.316	13.47	22.75	112	1.12	0.28	+7		
40	H.K.A...	June 5	60.0	2,400	0.321	8.2	20.9	87	1.00	0.32	-7		
41	2,333....	Apr. 20	73.6	1,240	0.321	18.85	23.35	120	1.17	0.27	+10		
42	L.S....	" 7	55.0	1,330	0.321	13.3	17.55	100	1.07	0.30	0		
43	R.A.S....	May 5	70.0	3,720	0.321	8.1	29.3	104	1.09	0.29	+3		
44	F.C.M...	" 21	77.2	816	0.321	24.8	20.1	112	1.14	0.28	+7		
45	F.C.M...	" 11	77.2	2,000	0.321	13.9	27.8	118	1.16	0.28	+7		
46	2,334....	Dec. 17	37.2	1,380	0.324	10.7	14.8	112	1.14	0.28	+7		Mitral stenosis.
47	2,365....	May 26	65.2	4,100	0.324	7.3	30.0	106	1.11	0.29	+3		
48	P.....	" 18	91.0	1,280	0.325	20.0	25.6	108	1.12	0.29	+3		
49	2,333....	Apr. 16	73.0	1,440	0.326	22.2	32.0	174	1.13	0.23	+23		
50	2,364....	" 20	46.6	1,285	0.326	16.85	21.6	160	1.38	0.24	+20		Exoph- thalmic goitre.
51	2,236....	Jan. 4	71.6	1,180	0.326	17.33	20.45	100	1.09	0.30	0		Aortic stenosis.
52	2,365....	May 13	61.0	1,700	0.326	11.7	19.9	94	1.06	0.31	-3		Aortic regurgi- tation.
53	2,316....	Jan. 27	71.0	1,620	0.328	15.7	25.5	110	1.20	0.27	+10		
54	2,365....	Mar. 8	59.0	462	0.330	27.0	12.5	91	1.06	0.31	-3		
55	2,316....	Apr. 1	76.2	900	0.336	20.6	18.4	87	1.05	0.32	-7		
56	2,333....	Mar. 8	69.0	1,760	0.336	17.45	30.7	147	1.36	0.25	+16		
57	2,098....	Dec. 1	71.0	870	0.336	20.4	17.75	90	1.06	0.32	-7		Auricu- lar fibril- lation.
58	F.C.M...	Apr. 28	80.0	1,240	0.341	18.7	23.2	96	1.12	0.30	0		
59	C.A.L...	May 12	84.0	1,000	0.342	19.6	19.6	80	1.02	0.34	-13		
60	A.A.G...	" 15	64.0	3,500	0.342	11.9	41.9	172	1.50	0.23	+23		
61	P.....	" 18	91.0	1,300	0.343	17.4	22.7	80	1.02	0.34	-13		
62	2,316....	Mar. 25	77.0	1,880	0.346	14.6	27.5	102	1.17	0.30	0		
63	2,316....	Apr. 9	76.8	2,240	0.346	14.9	33.3	126	1.30	0.27	+10		
64	2,333....	Mar. 29	71.0	2,080	0.346	15.8	32.9	138	1.36	0.25	+16		
65	2,375....	Nov 3	36.2	680	0.350	17.15	11.67	98	1.16	0.30	0		Mitral stenosis.
66	L.S....	May 19	55.0	1,056	0.353	24.8	26.3	171	1.54	0.23	+23		
67	2,316....	Mar. 8	75.2	3,280	0.356	8.88	29.1	84	1.07	0.33	-10		
68	2,316....	" 29	76.8	1,510	0.356	18.05	27.3	108	1.23	0.29	+3		

TABLE I.—*Concluded.*

No.	Subject.	Date.	Weight.	Urine per 24 hrs.	Urea.				$\sqrt{\frac{D}{Wt} \times \sqrt{C}}$	$\frac{Ur}{\sqrt{Wt}}$	$\sqrt{\frac{FD}{Wt} \times \sqrt{C}}$	Deviation from standard normal.	Remarks.
					Gm. per liter of blood Ur.	Gm. per liter of urine C.	Gm. per 24 hrs. D.	Index of excretion I.					
			<i>kilos</i>	<i>cc.</i>								<i>per cent</i>	
69	2,316...	Jan. 15	73.0	1,190	0.356	20.7	24.7	108	1.24	0.29	+ 3		
70	2,333...	Feb. 7	66.8	3,040	0.356	12.32	37.4	138	1.37	0.26	+13		
71	2,098...	Oct. 26	71.0	1,680	0.358	13.68	22.9	85	1.09	0.33	-10		
72	A.M.C...	May 6	67.5	1,250	0.361	21.2	26.5	125	1.34	0.27	+10		
73	R.A.S...	" 12	70.0	912	0.363	20.7	18.8	84	1.10	0.33	-10		
74	C.C...	Apr. 18	72.0	2,000	0.363	12.4	24.8	83	1.10	0.33	-10		
75	H.K.A...	June 4	60.0	1,100	0.363	21.8	23.9	126	1.37	0.29	+ 3		
76	F.C.M...	Apr. 29	77.2	960	0.363	31.2	29.3	144	1.46	0.25	+16		
77	2,301...	Mar. 26	55.0	595	0.366	23.2	13.8	84	1.10	0.33	-10		Post-pneu- monia.
78	L.S...	May 19	55.0	960	0.385	23.5	22.4	119	1.40	0.27	+10		
79	W.C.M...	" 18	61.0	2,800	0.385	11.9	33.3	112	1.37	0.28	+ 7		
80	R.E.P...	" 4	66.0	960	0.385	22.6	21.6	94	1.25	0.31	- 3		
81	H.U...	Apr. 18	45.0	750	0.385	20.1	15.1	91	1.23	0.31	- 3		
82	2,316...	Mar. 22	76.8	1,340	0.386	19.8	26.5	93	1.24	0.31	- 3		
83	C...	Jan. 10	70.0	860	0.386	25.6	22.0	96	1.26	0.31	- 3		
84	2,098...	Dec. 11	73.0	1,280	0.388	23.5	30.0	118	1.41	0.28	+ 7		
85	2,333...	Mar. 27	70.0	1,480	0.396	22.8	33.8	132	1.52	0.26	+13		
86	2,333...	Jan. 27	66.4	1,580	0.398	15.1	23.8	80	1.18	0.34	-13		
87	F.C.M...	May 28	77.2	2,424	0.406	25.2	63.3	224	2.03	0.20	+33		Urea 10 gm.
88	A.T...	Apr. 12	70.0	1,520	0.406	22.3	33.8	125	1.51	0.27	+10		
89	2,365...	Mar. 2	59.0	734	0.406	37.5	27.5	153	1.69	0.24	+20		
90	2,365...	Jan. 28	65.8	960	0.416	33.35	32.05	140	1.68	0.25	+16		
91	2,301...	Mar. 22	53.0	1,000	0.416	19.25	19.25	82	1.26	0.33	-10		
92	2,333...	" 22	70.8	1,780	0.427	20.5	36.6	116	1.53	0.28	+ 7		
93	R.A.S...	May 25	70.0	1,872	0.428	23.1	43.0	144	1.72	0.25	+16		
94	A.T...	" 21	70.0	1,368	0.428	20.9	20.0	90	1.36	0.31	- 3		
95	L.S...	" 23	55.0	1,176	0.428	29.9	23.7	96	1.40	0.31	- 3		
96	2,365...	Mar. 15	57.6	680	0.437	29.6	20.2	90	1.38	0.32	- 7		
97	2,364...	" 31	52.0	1,275	0.437	22.1	28.1	120	1.59	0.27	+10		
98	A.T...	May 15	70.0	1,200	0.449	24.1	28.6	89	1.42	0.32	- 7		
99	H.K.A...	" 22	60.0	988	0.470	24.8	24.3	82	1.42	0.33	-10		
100	2,190...	Oct. 16	53.2	780	0.470	27.5	21.5	86	1.45	0.32	- 7		Heart block.
101	2,190...	" 5	53.2	2,480	0.480	13.43	33.3	91	1.51	0.32	- 7		
102	W.C.M...	May 11	61.0	312	0.492	31.0	9.6	33	0.94	0.52	-73		
103	F.C.M...	" 25	77.2	1,536	0.513	35.1	52.6	138	2.01	0.26	+13		Urea 10 gm.
104	A.T...	" 28	70.0	3,400	0.513	23.9	81.3	194	2.38	0.22	+27		Urea 10 gm.
105	F.C.M...	" 18	81.6	5,400	0.539	15.7	84.7	122	2.02	0.27	+10		Urea 30 gm.
106	F.C.M...	" 18	81.6	4,320	0.542	16.75	72.5	111	1.90	0.28	+ 7		Urea 30 gm.
107	C.A.L...	" 25	84.0	1,100	0.577	31.6	34.8	63	1.53	0.38	-27		Urea 10 gm.

Examination of the tables shows in general an increase in the value of

$$\sqrt{\frac{D}{Wt}} \times \sqrt{C}$$

parallel with the increase in the concentration of urea in the blood. The result of this parallelism is to keep the formula

$$\frac{Ur}{\sqrt{\frac{D}{Wt}} \times \sqrt{C}}$$

relatively constant. The maximum deviations, tending toward an increase in rate of excretion, nearly all fall in those observations made when the blood urea figure was below 0.300 gram per liter. In this group a number of individuals show an index of 200 or more, corresponding to a deviation of +33 per cent from the normal laws. The probable reason for this is discussed below.

Particular interest, both from the physiological and clinical standpoint, attaches to the observations in which the blood urea is between 0.300 and 0.500 grams per liter. It is within these limits that a blood urea figure may be quite normal, or may occur with retention. Within these limits are 71 observations. Of these 71 observations, 52, or 73 per cent, lie within 10 per cent of the standard normal, corresponding to a range in the index from 84 to 125. 69, or 97 per cent, come within a range of 25 per cent of the standard normal. Of the two outside of this limit, one, previously reported, had apparent retention due to dehydration, the other was an experiment following the administration of urea by mouth. Out of the total of 107 observations, only 3 have an index below 80. Of these, Nos. 13 and 102 are directly attributable to the small amount of water ingested and the small amount of urine excreted, and such results are easily avoided in normal individuals by providing a sufficient amount of water before the observation. No. 107 followed ingestion of urea. An index of 80 is to be regarded as the lowest limit of normality, when the index is used as a measure of renal function.

Attention should be called to the conditions following the in-

gestion of large amounts of urea. In one case a concentration of 0.52 of a gram of urea per liter of blood was attained after 30 grams of urea, and in two observations the index was quite normal, the rate of excretion reaching at one time 84.7 grams of urea per twenty-four hours. We have not observed a concentration of urea in the blood above 0.500 in a normal individual under ordinary circumstances, and this concentration would be very difficult for a normal person to attain without ingestion of urea, on account of the very rapid rate of excretion which takes place when the concentration in the blood approaches this level.

Since we have observed blood urea figures between 0.300 and 0.500 about thirty times in perfectly normal individuals under usual conditions of diet, etc., there can be no question that such figures are within the usual range. During the same time we have found the blood urea below 0.300 only twenty-three times in the same class of individuals. Since the laws of excretion hold very closely for the range from 0.300 and 0.500 and show greater deviations below 0.300, it appears that the lower figures in the blood are due to a rate of excretion, which, while not in any sense abnormal, is higher than that shown by the majority of individuals. These figures occur mainly in young, active adults. It should be noted that the variations in the blood urea figure, as well as in the index, are as great for single individuals as they are for all normals.

We may summarize the findings regarding normal urea excretion as follows: The normal concentration of urea in the blood varies from about 0.200 to 0.500 grams per liter, in the same or different individuals. The rate of excretion is determined by this concentration and by the rate of water output. The laws given above hold closely for concentrations of urea in the blood between 0.300 and 0.500 grams per liter, and somewhat less so for concentrations below 0.300, the tendency in the latter case being toward a higher rate of excretion. Findings simulating those of urea retention are easily avoided by providing for a sufficient output of urine, by administering water before the period of observation.

Normal Chloride Excretion.

Table II summarizes the results obtained from observations on chloride excretion. The class of individuals used and criteria of selection were the same as those in Table I, many of the observations in the two tables being made simultaneously. In this table we have arranged the observations in order according to the calculated value of plasma sodium chloride from the formula given, and have compared in each case the concentration actually found. The difference is expressed as + or —, according to whether the concentration actually found was higher or lower than the concentration calculated from the rate of excretion. We have also calculated the threshold in each case, *i. e.*, the threshold to which

$$\sqrt{\frac{D \sqrt{C}}{4.23 \times Wt}}$$

being added will give the concentration actually found in the plasma. It will be seen that on the whole there is a remarkably close agreement between the actual and calculated plasma sodium chloride, and this agreement is usually within the limit of error in determining the plasma sodium chloride. The threshold is therefore very constant at about 5.62 grams per liter, as originally stated by Ambard and Weill. The actual average of all values for threshold in the table is 5.61 grams +. A certain amount of deviation, as yet unexplained, must be recognized. The maximum variations observed are from 5.24 to 5.84 grams. 65 observations, or 90 per cent, are within a range of 5.52 to 5.72 grams. 50, or 70 per cent, are within range of 5.57 to 5.67 grams, or within range of experimental error of the average threshold of 5.62 grams.

The deviations from the threshold have occurred mainly in young active individuals. It has been a striking feature, not alone in the figures presented, that hospital patients with normal excretion, leading well regulated lives, have an average greater degree of constancy in the chloride threshold and excretion than strictly normal, active individuals.

As previously mentioned, Ambard attributes certain changes in the threshold to the relation of diet, time of meals, etc. Table III

TABLE II *

The Relation of the Rate of Chloride Excretion (Calculated as Sodium Chloride) to Concentration in Plasma in 72 Observations in Individuals with Normal Excretion, Arranged according to Rate of Excretion, as Modified by Concentration in Urine, and Expressed as Calculated Sodium Chloride in Plasma.

$$\text{Calculated plasma NaCl} = 5.62 + \sqrt{\frac{\text{Gm. per 24 hrs. } \sqrt{\text{Gm. per liter}}}{\text{Wt. in kilos} \times 4.23}}$$

$$\text{Threshold} = \text{actual plasma NaCl} - \sqrt{\frac{\text{Gm. per 24 hrs. } \sqrt{\text{Gm. per liter}}}{\text{Wt. in kilos} \times 4.23}}$$

No.	Subject.	Date.	Weight.	Urine 24 hrs.	Blood urea per liter.	Index of urea excre- tion I.	Sodium chloride.				Remarks.		
							Gm. per liter of urine C.	Gm. per 24 hrs. D.	Calcu- lated.	Actual.			
			<i>kilos</i>	<i>cc.</i>	<i>gm.</i>								
1	2,365.....	Mar. 8	59.0	462	0.330	91	2.3	1.18	5.70	5.67	-.03	5.59	Aortic regurgitation.
2	2,365.....	" 2	59.0	734	0.406	153	4.7	3.45	5.78	5.73	-.05	5.57	
3	V.S.....	May 5	72.0	2,300	0.191	170	3.1	7.5	5.82	5.81	-.01	5.61	
4	2,365.....	Mar. 15	57.6	680	0.437	90	6.2	4.23	5.83	3.83	0	5.62	Post-pneumonia.
5	2,304.....	Apr. 20	46.6	1,285	0.326	160	3.7	4.75	5.84	5.81	-.03	5.59	
6	2,195.....	Nov. 24	53.2	1,220			4.0	4.9	5.85	5.90	+ .05	5.67	
7	2,181.....	" 16	45.0	1,100			4.7	5.17	5.86	5.81	-.05	5.57	Aortic regurgitation.
8	2,316.....	Jan. 15	73.0	1,190	0.356	108	6.9	8.21	5.88	5.91	+ .03	5.65	
9	F.C.M.....	May 4	80.0	3,400	0.254	164	3.7	12.6	5.89	6.05	+ .16	5.78	
10	F.C.M.....	Jan. 20	80.0	1,073	0.221	191	8.4	9.02	5.90	5.94	+ .04	5.66	Auricular fibrillation.
11	L.L.V.S.....	May 10	58.0	2,360	0.301	75	4.0	9.45	5.90	5.89	-.01	5.61	
12	2,198.....	Dec. 1	71.0	870	0.336	90	9.5	8.26	5.91	5.90	-.01	5.61	
13	E.S.....	Mar. 19	88.0	730	0.316	110	12.1	8.75	5.91	5.88	-.03	5.59	Exophthalmic goitre.
14	2,364.....	" 31	52.0	1,275	0.437	120	6.0	7.65	5.91	5.87	-.04	5.58	
15	V.S.....	May 5	72.0	5,100	0.224	95	2.9	14.8	5.91	5.87	-.04	5.58	
16	V.S.....	" 18	72.0	600	0.232	70	13.1	7.86	5.93	5.95	+ .02	5.64	Mitral stenosis (digitalis).
17	2,337.....	Jan. 23	39.8	780	0.244	308	7.9	6.16	5.93	5.95	+ .02	5.64	

* Those with initials italicized were healthy normal adults. The numbers are hospital records.

No.	Subject.	Date.	Weight. kilos	Urine per 24 hrs.	Blood urea per liter.	Index of urea excretion I.	Sodium chloride.					Remarks.	
							Gm. per liter of urine C.	Gm. per 24 hrs. D.	Gm. per liter of plasma.				
									Calcu- lated.	Actual.	Differ- ence.		
18	F.C.M.	May 5	80.0	2,560	cc.	196	5.3	13.6	5.93	6.15	+0.22	5.84	Aortic regurgitation.
19	2,198	Dec. 11	73.0	1,280	gm.	117	8.7	11.14	5.94	5.95	+0.01	5.63	
20	H.T.C.	Mar. 19	65.0	1,282		190	7.9	10.1	5.94	5.93	-0.01	5.61	
21	F.C.M.	Oct. 6	78.0	1,120		152	9.2	10.3	5.96	5.95	-0.01	5.61	
22	A.R.D.	May 5	67.0	1,560		170	7.5	11.7	5.96	6.08	+0.12	5.74	Aortic regurgitation.
23	E.S.	" 19	86.0	1,295		153	11.1	14.4	5.98	6.05	+0.07	5.69	
24	2,333	Jan. 27	66.4	1,580		80	8.2	12.95	5.98	6.00	+0.02	5.64	
25	2,316	Feb. 16	73.2	1,000		100	12.4	12.4	4.99	5.96	-0.03	5.59	
26	F.C.M.	Mar. 19	80.0	2,462			7.0	17.25	5.99	6.04	+0.05	5.67	Aortic regurgitation.
27	V.S.	May 18	72.0	736		122	15.5	11.4	6.00	5.95	-0.05	5.57	
28	2,236	Jan. 4	71.6	1,180		100	11.4	13.45	6.00	5.95	-0.05	5.57	
29	A.R.D.	May 5	67.0	2,820		128	5.9	16.7	6.00	6.12	+0.12	5.74	
30	2,365	" 13	61.0	1,700		94	7.7	13.1	6.00	5.94	-0.06	5.56	"
31	2,305	" 26	65.2	4,100		106	4.5	18.4	6.00	5.94	-0.06	5.56	
32	F.C.M.	" 18	81.6	4,320		111	5.3	22.9	6.01	5.94	-0.07	5.55	Urea 30 gm.
33	2,333	Mar. 1	68.4	920		91	13.3	12.22	6.01	5.99	-0.02	5.60	
34	2,316	" 25	77.0	1,880		102	8.7	16.35	6.01	5.99	-0.02	5.60	Auricular fibrillation. Post-pneumonia.
35	2,333	Apr. 30	72.2	1,860		176	8.6	16.0	6.01	6.00	-0.01	5.61	
36	A.M.C.	May 19	68.2	4,850		123	4.5	21.8	6.02	5.86	-0.16	5.46	
37	F.C.M.	" 17	81.6	2,880		220	7.4	20.7	6.03	6.02	-0.01	5.61	
38	2,337	Jan. 19	62.0	1,160		230	8.7	10.1	6.03	6.05	+0.02	5.64	
39	2,351	Apr. 26	68.5	3,660		84	3.9	14.6	6.03	5.99	-0.04	5.58	
40	2,175	Nov. 9	49.0	700			13.5	9.45	6.03	6.02	-0.01	5.61	
41	2,365	Apr. 4	59.8	1,460		102	9.5	13.9	6.03	6.01	-0.02	5.60	
42	E.S.	May 19	86.0	1,125		228	15.0	16.9	6.04	5.95	-0.09	5.53	
43	2,365	" 27	65.2	1,940		155	8.7	16.9	6.04	5.95	-0.09	5.53	
44	2,181	Nov. 24	43.4	1,120			9.4	10.5	6.04	6.02	-0.02	5.60	
45	2,365	Jan. 28	65.8	960		140	13.7	13.15	6.04	6.04	0	5.62	
46	F.C.M.	Mar. 18	80.0	1,380		159	12.8	17.65	6.05	6.11	+0.06	5.68	

Aortic regurgitation.

Aortic regurgitation.

"

Urea 30 gm.

Auricular fibrillation.
Post-pneumonia.

TABLE III.*
Observations Made on the Same Individuals, on the Morning and Afternoon of the Same Day.
All Were Normal, Healthy Adults.

No.	Subject.	Time.	Date.	Weight.	Urine per 24 hrs.	Blood per liter.	Index of urea excre- tion I.	Sodium chloride.				Remarks.	
								Gm. per liter of urine C.	Gm. per 24 hrs. D.	Calcu- lated.	Actual.		
				<i>kilos</i>	<i>cc.</i>	<i>gm.</i>							
1	F.C.M.....	a.m.	Apr. 28	80.0	1,240	0.341	96	14.5	18.0	6.07	6.10	+0.03	5.65
		p.m.	" "	80.0	2,140	0.266	182	17.3	37.0	6.30	6.40	+0.10	5.72
2	F.C.M.....	a.m.*	May 5	80.0	1,760	0.206	220	10.9	19.2	6.05	6.20	+0.15	5.77
		p.m.	" "	80.0	2,560	0.206	196	5.3	13.6	6.93	6.15	+0.22	5.84
3	A.R.D.....	a.m.	" "	67.0	1,560	0.206	170	7.5	11.7	5.96	6.08	+0.12	5.74
		p.m.	" "	67.0	2,820	0.226	128	5.9	16.7	6.00	6.12	+0.12	5.74
4	V.S.....	a.m.	" "	72.0	5,100	0.224	95	2.9	14.8	5.91	5.87	-0.04	5.58
		p.m.	" "	72.0	2,300	0.191	170	3.1	7.5	5.82	5.81	-0.01	5.61
5	F.C.M.....	a.m.	" 18	81.6	5,400	0.539	122	5.4	29.2	6.06	5.97	-0.09	5.53
		p.m.	" "	81.6	4,320	0.542	111	5.3	22.9	6.01	5.94	-0.07	5.55
6	V.S.....	a.m.	" "	72.0	600	0.232	70	13.1	7.86	5.93	5.95	+0.02	5.64
		p.m.	" "	72.0	736	0.228	122	15.5	11.4	6.00	5.95	-0.05	5.57
7	P.....	a.m.	" "	91.0	1,300	0.343	80	18.7	24.3	6.14	6.04	-0.10	5.52
		p.m.	" "	91.0	1,280	0.325	108	16.3	20.8	6.09	5.99	-0.10	5.52
8	A.M.C.....	a.m.	" 19	68.2	4,850	0.280	123	4.5	21.8	6.02	5.86	-0.16	5.46
		p.m.	" "	68.2	1,125	0.261	175	10.8	28.0	6.16	5.78	-0.38	5.44
		a.m.	" "	86.0	1,125	0.212	228	15.0	16.9	6.04	5.95	-0.09	5.53
9	E.S.....	p.m.	" "	86.0	1,295	0.220	153	11.1	14.4	5.98	6.05	+0.05	5.67
													Sodium chloride 10 gm.
													Urea 30 gm.
													" "

* The calculations are the same as in Table II.

shows the findings from observations made in the same individuals just preceding and two to three hours after a heavy noon meal. This table includes the maximum variations in the threshold that have been found, in both directions. Out of nine observations, in only two, Nos. 8 and 9, is there evidence of any appreciable change in the threshold between the morning and afternoon periods. In one of these the threshold was already very low in the morning and was still lower in the afternoon. In No. 9 the threshold was apparently raised between the two periods. In none of the cases was any particular attention paid to diuretics, most of the subjects drinking tea with the noon meal.

The individual variations in the chloride threshold and excretion are also of interest. Table IV contains the results of numerous observations made in the same individuals and shows the range of variations that may be encountered under conditions that are approximately identical. Nos. 1, 2, 3, and 4 are normal individuals; 5, 6, and 7 are cardiac cases with full compensation. Nos. 5 and 7 include some observations in subjects under the influence of digitalis; No. 6 includes only the observations made when the patient was not under the influence of any drugs. Subject 1, in whom the greatest number of observations was made, shows the maximum variation. There was a tendency toward a high threshold at most times, and a lowered threshold occurred only under the diuretic effects of large doses of urea.

A comparison of the chloride results with the blood urea figures and the index of urea excretion discloses no interrelationship between the two functions. Either a high or a low threshold for chlorides may exist coincidently with a high or low blood urea or high or low index. No evidence has been found which causes us to believe that the normal variations in chloride and urea functions are parallel. The degree of constancy of chloride excretion is more striking than that of urea, and remains so in the presence of the maximum variations in urea function.

The findings regarding normal chloride excretion may be summarized as follows: The normal and usual range of concentration of chlorides in human plasma is from 5.62 to 6.25 grams of sodium chloride per liter or higher, according to the amount ingested. On

TABLE IV.*
Repeated Observations on the Same Individuals, to Show Normal Variations.

No.	Subject.	Date.	Weight.	Urine per 24 hrs.	Blood urea per liter.	Index of excre- tion I.	Sodium chloride.					Remarks.	
							Gm. per liter of urine C.	Gm. per 24 hrs. D.	Gm. per liter of plasma.		Thresh- old.		
									Calcu- lated.	A ctua l.			
1†	F.C.M.....	1914 Oct. 6	kilos 78.0	cc. 1,120	gm. 0.298	152	9.2	10.3	5.96	5.95	-01	5.61	Sodium chlo- ride 10 gm.
	Normal	1915 Jan. 20	80.0	1,073	0.221	191	8.4	9.02	5.90	5.94	+04	5.66	
		Mar. 18	80.0	1,380	0.286	159	12.8	17.65	6.05	6.11	+06	5.68	
		" 19	80.0	2,462			7.0	17.25	5.99	6.04	+05	5.67	
		Apr. 28, a.m.	80.0	1,240	0.341	96	14.5	18.0	6.07	6.10	+03	5.65	
		p.m.	80.0	2,140	0.266	182	17.3	37.0	6.30	6.40	+10	5.72	
		May 4	80.0	3,400	0.254	164	3.7	12.6	5.89	6.05	+16	5.78	
		" 5, a.m.	80.0	1,760	0.206	220	10.9	19.2	6.05	6.20	+15	5.77	
		p.m.	80.0	2,560	0.206	196	5.3	13.6	5.93	6.15	+22	5.84	
		" 17	81.6	2,800	0.211	220	7.4	20.7	6.03	6.02	-01	5.61	
		" 18, a.m.	81.6	5,400	0.539	122	5.4	29.2	6.06	5.97	-09	5.53	Urea 30 gm. " " "
		p.m.	81.6	4,320	0.542	111	5.3	22.9	6.01	5.94	-07	5.55	
2†	V.S.....	" 5, a.m.	72.0	5,100	0.224	95	2.9	14.8	5.91	5.87	-04	5.58	
	Normal	p.m.	72.0	2,300	0.191	170	3.1	7.5	5.82	5.81	-01	5.61	
		" 18, a.m.	72.0	600	0.232	70	13.1	7.86	5.93	5.95	+02	5.64	
		p.m.	72.0	736	0.228	122	15.5	11.4	6.00	5.95	-05	5.57	
3†	A.M.C.....	" 6	67.5	1,250	0.361	125	13.0	16.25	6.07	6.16	+09	5.71	
	Normal.....	" 19, a.m.	68.2	4,850	0.280	123	4.5	21.8	6.02	5.86	-16	5.46	
		p.m.	68.2	1,125	0.261	175	10.8	28.0	6.16	5.78	-38	5.24	
4†	E.S.....	Mar. 19	88.0	730	0.316	110	12.1	8.75	5.91	5.88	-03	5.58	
	Normal	May 19, a.m.	86.0	1,125	0.212	228	15.0	16.9	6.04	5.95	-09	5.53	
		p.m.	86.0	1,295	0.220	153	11.1	14.4	5.98	6.05	+07	5.69	

* The calculations are the same as in Table II.

† Nos. 1, 2, 3, and 4 are healthy normal adults.

TABLE IV.—*Concluded.*

No.	Subject.	Date.	Weight.	Urine per 24 hrs.	Blood urea per liter.	Index of urea excre- tion I.	Sodium chloride.			Remarks.	
							Gm. per Gm. per liter of urine C.	Gm. per liter of plasma.	Thresh- old.		
							Calcu- lated.	Actual.	Differ- ence.		
5†	2,365 Aortic regurgitation	1915	kilos	cc.	gm.						
		Jan. 28	65.8	960	0.416	140	13.7	13.15	6.04	6.04	0 5.62
		Feb. 24		1,140	0.344	182	9.9	11.3	5.96	5.72	-24 5.38
		Mar. 2		734	0.406	153	4.7	3.45	5.78	5.73	-05 5.57
		" 8	59.0	462	0.330	91	2.3	1.18	5.70	5.67	-03 5.59
		" 15	57.6	680	0.437	90	6.2	4.23	5.83	5.83	0 5.62
		May 4	49.8	1,460	0.301	102	9.5	13.9	6.03	6.01	-02 5.60
		" 5	59.8	1,900	0.296	116	9.2	17.5	6.08	5.82	-26 5.36
		" 13	61.0	1,700	0.326	94	7.7	13.1	6.00	5.94	-06 5.56
		" 26	65.2	4,100	0.324	106	4.5	18.4	6.00	5.94	-06 5.56
		" 27	65.2	1,940	0.304	155	8.7	16.9	6.04	5.95	-09 5.53
6†	2,333 Aortic regurgitation	Jan. 27	66.4	1,580	0.398	79	8.2	12.95	5.98	6.00	+02 5.64
		Feb. 7	66.8	3,040	0.356	108	7.1	21.6	6.06	6.04	-02 5.60
		Mar. 1	68.4	920	0.276	91	13.3	12.22	6.01	5.99	-02 5.60
		" 8	69.2	1,760	0.336	147	13.2	23.3	6.16	6.19	+03 5.65
		" 22	70.8	1,780	0.427	128	13.0	23.2	6.15	6.11	-04 5.58
		Apr. 16	73.0	1,440	0.326	174	14.2	20.4	6.12	6.15	+03 5.65
		" 20	73.6	1,240	0.321	120	12.8	15.9	6.05	5.98	-07 5.55
		" 30	72.2	1,860	0.316	176	8.6	16.0	6.01	6.00	-01 5.61
		Jan. 19	42.0	1,160	0.195	230	8.7	10.1	6.03	6.05	+02 5.64
		" 23	39.8	780	0.244	308	7.9	6.16	5.93	5.95	+02 5.64
		" 28	39.6	660	0.266	210	6.0	4.0	5.86	5.91	+05 5.67
7†	2,337 Mitral stenosis	Feb. 26	41.0	1,100	0.246	153	12.4	13.6	6.15	6.23	+08 5.70
		Mar. 1	41.0	1,300	0.184	240	11.44	14.88	6.16	6.15	-01 5.61
		" 22	43.0	1,110	0.256	180	11.0	12.2	6.09	6.08	-01 5.61
		Apr. 30	40.6	1,640	0.251	216	8.0	13.1	6.08	6.08	0 5.62

† Nos. 5, 6, and 7 are cases with cardiac lesions but with normal excretion.

the excess over a threshold of about 5.62 grams per liter depends the rate of excretion, the laws governing which may be expressed numerically. A concentration below 5.62 grams per liter has not been observed in a normal individual.

DISCUSSION.

In the above laws for both urea and sodium chloride four variable factors are considered; namely, the concentration in blood and urine, the rate of excretion, and the weight of the individual. Indirectly the water content of the blood is considered; inasmuch as it influences the rate of water output and thereby the concentration of urea or sodium chloride in the urine, and the rate of their excretion. The use of body weight in the formula implies that the amount of blood flowing through the kidneys in a given time is relatively constant per kilo of body weight, and also that the amount of functioning kidney tissue is in direct proportion to the body weight. That the factors of blood flow, etc., are subject to variations from time to time, in the same individual, as well as in different individuals, can not be doubted. That the normal variations in the laws are due to such changes can not at present be shown, but it seems that if these factors could be controlled, the degree of constancy might be much higher. Inasmuch as the variations in different individuals of far different weights and proportions are no greater than the variations in the same individual at different times, the relation of body weight to the rate of excretion seems well established. Body temperature has been shown by Ambard to be of some importance, but the normal variations are apparently not sufficient to influence the rate of excretion. That such a number of possible factors influencing a physiological process, such as the rate of excretion of urea or sodium chloride, can be controlled and brought into numerical laws which show a high degree of constancy is of great importance from the standpoint of normal and pathological physiology. It suggests that many of the so called vital processes may work under laws as definite as the better known laws of physics. The rate of flow of liquids under different conditions has long been subject to numerical expression by the laws of hydrostatics. That

at least certain functions of body tissues can be as well defined now appears probable.

CONCLUSIONS.

1. The excretion of urea and of chlorides in the normal individual is carried out according to definite laws, capable of numerical expression.

2. The rate of excretion of urea and of sodium chloride is determined by the concentration in the blood, the rate of water output, and indirectly by the weight of the individual.

3. The threshold of sodium chloride excretion is practically constant at about 5.62 grams of sodium chloride per liter of plasma. Slight variations in the threshold occur in normal individuals.

4. The rate of excretion of urea under the conditions found at any time can be measured directly in terms of the normal by an index of urea excretion.

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EXPLANATION OF PLATES.

Figs. 1 and 2 show a slide-rule adapted to calculation of urea and sodium chloride formulas.

PLATE 27.

FIG. 1. I, II, and III show the calculation of the urea index.

Example.

Gm. urea excreted per 24 hrs., $D = 20.0$
 Gm. urea per liter of urine, $C = 11.0$
 Gm. urea per liter of blood, $Ur = 0.330$
 Body weight in kilos, $Wt = 55.0$

I. 55.0 on Wt scale is set opposite 20.0 on D scale (first position).

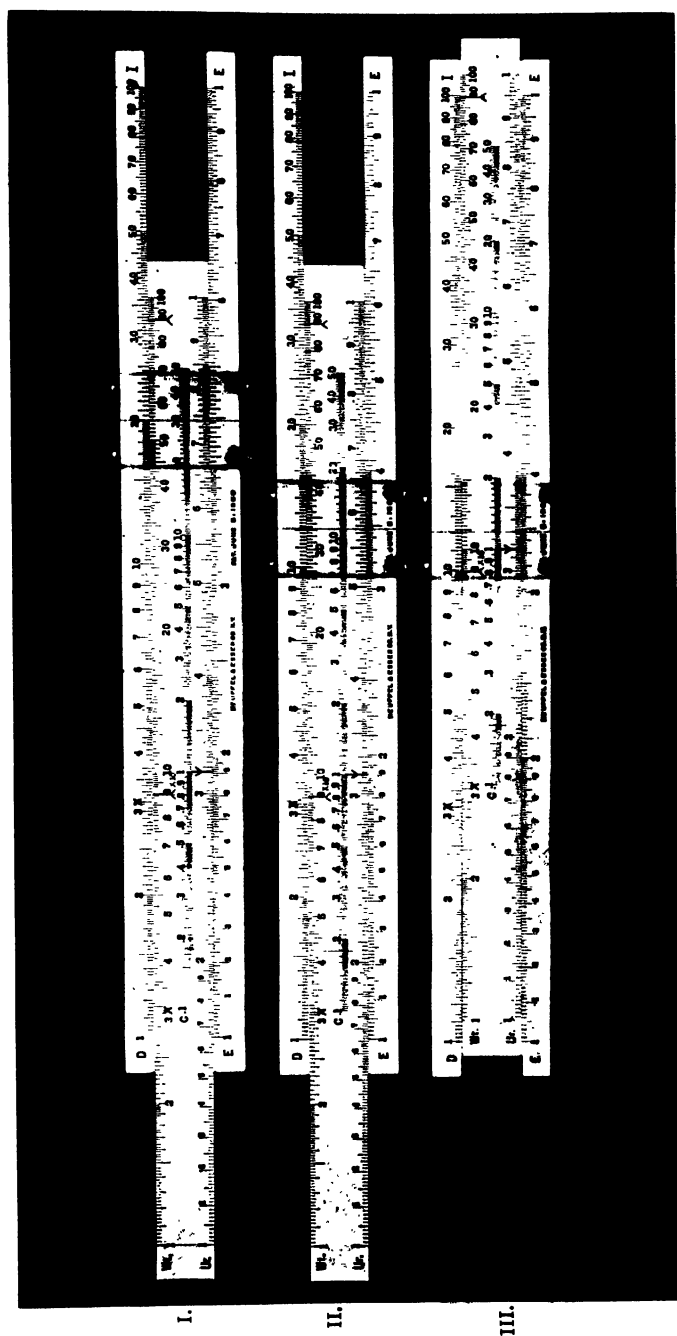


FIG. 1.

(McLean: Laws Governing Rate of Excretion in Man.)

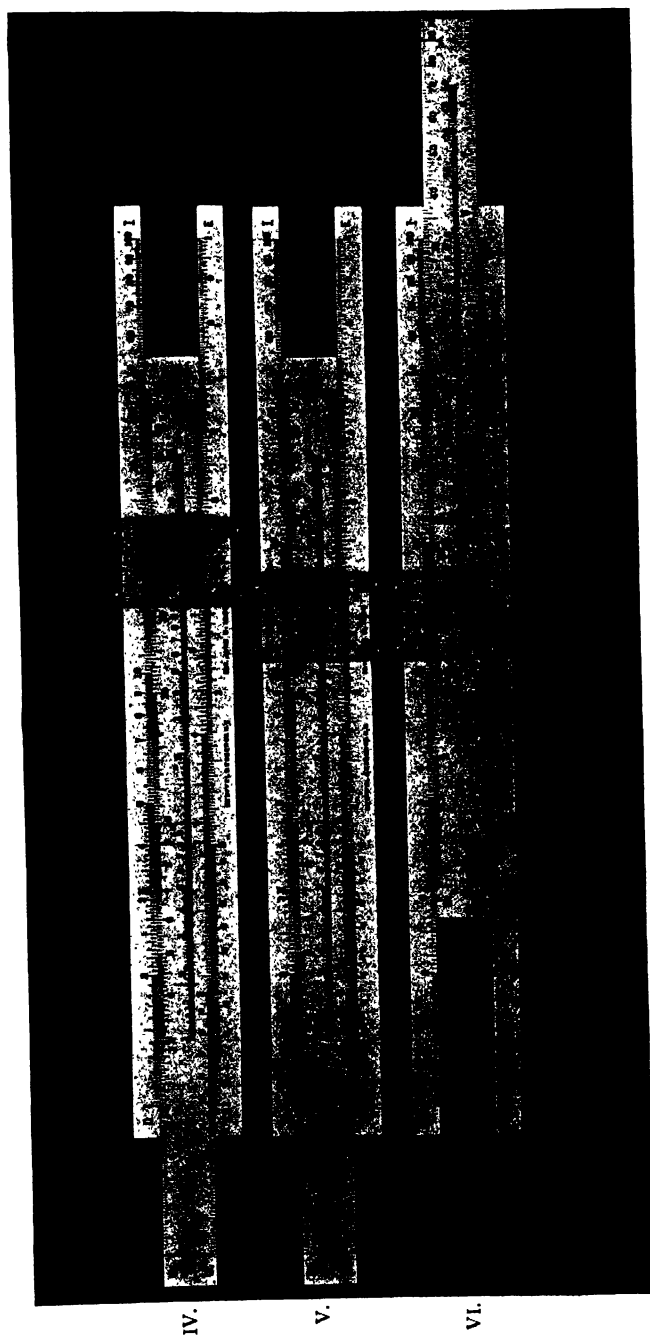


FIG. 2.

(McLean: Laws Governing Rate of Excretion in Man.)

- II. Hair line on runner is moved to 11.0 on C scale (second position).
 III. Slide is moved so that 3.30 on Ur scale is at hair line on runner (third position).
 Reading is now made at the arrow which points to scale I and is between 99.0 and 100.0. Therefore the index, I, is 100.0.

PLATE 28.

FIG. 2. IV, V, and VI show the calculation of plasma sodium chloride.

Example.

Gm. sodium chloride excreted per 24 hrs., $D = 18.0$
 Gm. sodium chloride per liter of urine, $C = 9.0$
 Body weight in kilos, $Wt = 40.0$

- IV. 40.0 on Wt scale is set opposite 18.0 on D scale (first position).
 V. Hair line on runner moved to 9.0 on C scale (second position).
 VI. Constant at 4.23 on Wt scale moved to hair line on runner.
 Reading is made opposite the arrow on E scale, which is at 0.57.
 Calculated plasma sodium chloride = $5.62 + E = 5.62 + 0.57 = 6.19$.

THE CONCENTRATION OF THE PROTECTIVE BODIES IN ANTIPNEUMOCOCCUS SERUM. SPECIFIC PRECIPITATE EXTRACTS.

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It has been noted in a previous communication (1) that the immune substances in antipneumococcus serum may be removed by specific precipitation with extracts of the pneumococcus. It was further shown that the precipitates, when suspended in normal salt solution, and to a less extent when dissolved in a weak solution of sodic hydrate, protect susceptible animals against many times the lethal dose of pneumococcus. The present work is a further study of the action of the precipitates as well as of extracts of precipitates made by various methods.

An attempt was made to render soluble the specific precipitates by the use of weak alkaline salts. As in the preceding experiments (1), it appeared that the amount of sodic hydrate necessary to dissolve the precipitates was so great that in many cases the solution proved either toxic for animals or had suffered diminution in protective properties. Such weak alkalis as sodium phosphate, sodium biphosphate, and sodium carbonate were not strong enough to cause a solution of the precipitate. However, it was noted that when sodium carbonate was added to an emulsion of the whole precipitate in salt solution, a definite flocculation of the suspended particles occurred, and the flocculated particles quickly settled down, leaving an opalescent supernatant fluid which contained protective bodies, agglutinins, and precipitins. This observation suggested the possibility of bringing about a dissociation of the antigen and antibody of the specific precipitate.

It has been shown by Pfeiffer and Friedberger, and Bail and his pupils that the formation of the antigen-antibody complex that takes place in the precipitate

which forms when the appropriate bacterial precipitinogen is added to an immune serum, is relatively a loose one, and can be dissociated subsequently by extraction in salt solution. In 1903 Pfeiffer and Friedberger (2) found that when thoroughly washed sensitized cholera vibrios were injected into the peritoneal cavity of a guinea pig, the guinea pig was able to survive a second lethal dose of fresh live cholera vibrios, when injected one or two hours later. This work has been subsequently confirmed and elaborated by Landsteiner and Jagic (3), Hoke (4), Bail and Rotky (5), Bail (6), and Bail and Tsuda (7); and more recently Krauss (8) and Matsui (9) have demonstrated this dissociation of antigen and antibody, both *in vivo* and *in vitro*. They precipitated the immune substances in normal beef serum with live cholera vibrios and with *Bacillus typhosus*, and determined the optimum relation of culture to serum and the effects of varying degrees of temperature on the formation of the precipitate and on the potency of the extracts obtained from such precipitates. In many plate experiments the bactericidal or inhibiting action of these extracts on the growth of cholera vibrios has been demonstrated.

In the present work it will be shown that extracts of the precipitates formed in antipneumococcus serum by the addition of bacterial precipitinogen not only exert an inhibiting influence on the growth of virulent pneumococci *in vitro*, but the extracts contain agglutinins and precipitins and protect susceptible animals, such as the mouse and rabbit, as efficiently as does the original antipneumococcus serum. On the other hand, these extracts contain only a minimal amount of protein as compared with the whole serum. Moreover, considerable experimental evidence suggests that the extracts, and more especially the whole precipitates, produce active as well as passive immunity to pneumococcus infection in mice.

Methods.

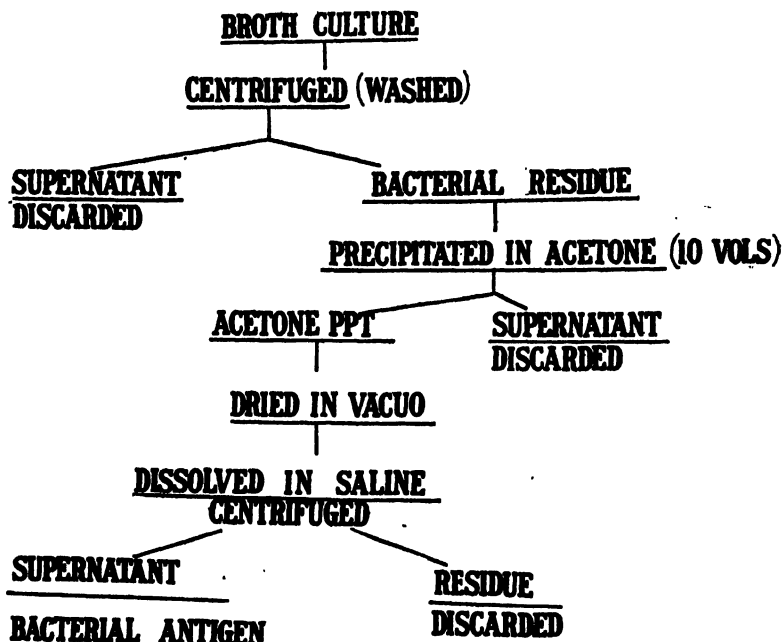
As certain modifications have been made in the methods described in the first communication, the preparation of the bacterial precipitinogen and the method of precipitating the serum are reviewed in detail. For instance, acetone instead of alcohol has been used exclusively to kill the bacteria used in the preparation of the extract. Methods have also been employed for the more complete exhaustion of the immune serum.

Preparation of Pneumococcus Extracts (Precipitinogen).

An outline of the method is shown in Text-fig. 1. A twenty-four hour plain broth culture of pneumococcus of Type I or Type

II (10) is centrifuged and the bacterial residue washed twice in normal salt solution. The washed bacterial residue of a liter of broth culture is then emulsified in 5 cc. of normal salt solution and is added slowly to 10 volumes of acetone. There is an immediate flocculation of the bacteria. The mixture is then quickly centrifuged within five minutes, and the supernatant acetone decanted. The bacterial residue is then quickly dried *in vacuo*. The average

PREPARATION OF PNEUMOCOCCUS ANTIGEN



TEXT-FIG. 1.

yield of dried bacteria from a liter of broth is about 100 to 150 mg. The acetone-killed bacteria may be stored in dry form until ready for use in precipitating the immune serum.

The dried bacteria are dissolved in normal salt solution, usually 1 to 2 mg. per cc. On shaking thoroughly, the solution of the bacteria is rapid and almost complete. After the bacteria have been dissolved as completely as possible, the solution is centrifuged at high

speed for one-half hour. Usually a very small amount of sediment collects at the bottom of the tube. The opalescent supernatant fluid is used as the bacterial extract in precipitating the immune substances from antipneumococcus serum.

Since acetone does not kill the ferments (Van Slyke and Cullen¹ (11)) these bacterial extracts may undergo autolysis. Hence it is advisable to store the bacterial precipitinogen in dry form from which fresh extracts can be readily prepared when needed. However, saline extracts of the bacteria have been used after storage on ice for two months and have shown no appreciable decrease in their ability to precipitate immune sera.

Acetone kills the pneumococcus probably by very rapid dehydration. Cultures of these bacterial extracts are always sterile when the procedure is carried out with aseptic precautions. Smears of the bacterial extracts show a Gram-negative staining amorphous material. Kjeldahl determinations made under Mr. Cullen's direction show that the bacterial extract is practically pure protein, as it contains 16.2 per cent nitrogen.

Method of Specific Precipitation of Antipneumococcus Serum.

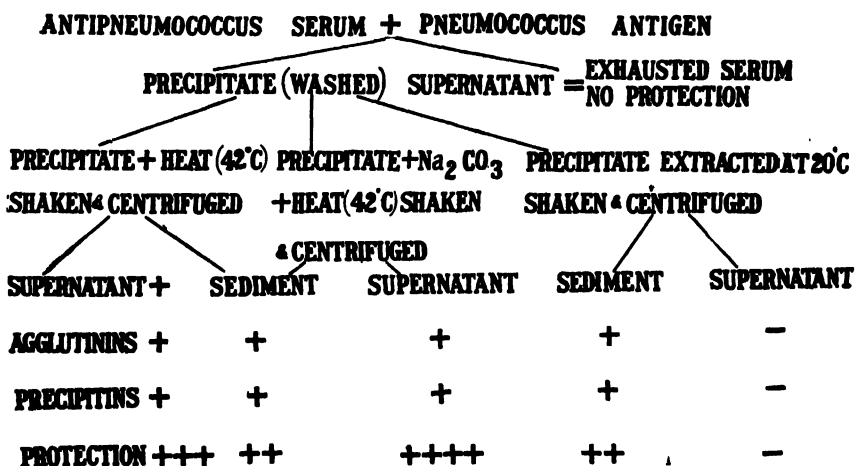
An outline of the methods employed, showing the method of obtaining the various fractions, is shown in Text-fig. 2. The presence or absence of immune bodies in the various fractions is also indicated. These facts are shown in detail later.

To an antipneumococcus serum, prepared by actively immunizing a horse to live cultures of pneumococcus of Type I or Type II, a bacterial extract of pneumococcus of the corresponding group is added until the immune serum is exhausted of its antibodies. Testing the absence of agglutinins in the serum from which the precipitate has been removed is a more accurate criterion of complete exhaustion of the serum than is the repeated addition of bacterial precipitinogen until no further precipitate occurs. By this method it is possible to exhaust completely the serum of its protective properties. To exhaust antipneumococcus serum it is necessary to add

¹ Buchner was the first to note that acetone did not kill the oxidases. Van Slyke, having prepared an active form of urease by precipitation with acetone, suggested its use in the preparation of pneumococcus extracts.

on the average the bacterial extract of 50 mg. dry weight of bacteria per 100 cc. of serum. It makes little difference, as regards the total amount of bacterial extract necessary to exhaust a given quantity of serum, whether the precipitinogen be added at once or repeatedly in small amounts. The precipitate formed by the addition of the bacterial extract as precipitinogen is apparent at once and is very volu-

SPECIFIC PRECIPITATION OF ANTIBODIES FROM ANTIPNEUMOCOCCUS SERUM



TEXT-FIG. 2.

minous. The precipitated serum is incubated at 38° C. for two hours to render the reaction complete and is then stored on ice over night, whereupon it is centrifuged. The supernatant serum fluid is pipetted off and is called exhausted serum. The precipitate is washed three times in normal salt solution, in order to free it completely of serum, and is then ready for extraction.

Methods of Extracting the Protective Substances from Specific Precipitates of Antipneumococcus Serum.

Extraction in Normal Salt Solution at 20° C.—The washed specific serum precipitate is emulsified in normal salt solution in one-half to one-fifth the volume of the original serum. It is allowed to

stand at room temperature for twenty-four hours, being shaken occasionally. In the following experiments this extract is designated saline extract. This preparation is unsatisfactory, as the extract does not protect animals as highly as do extracts prepared by the methods described below.

Extraction in Normal Salt Solution at 42° C.—The washed serum precipitate is suspended in normal salt solution as described above. It is then heated for one hour at 42° C., being shaken gently at intervals, after which it is centrifuged at low speed. The supernatant fluid from this heated saline emulsion is called heat extract. This extract contains agglutinins and precipitins, and protects mice against many times the lethal dose of pneumococcus.

Extraction in Normal Salt Solution at 42° C. with Sodium Carbonate.—The washed serum precipitate is suspended in salt solution as described above, and about 1 cc. of a 1 per cent sodium carbonate solution is added to the precipitate from 100 cc. of serum, or an amount sufficient to flocculate the suspended particles of precipitate. The flocculated emulsion is then heated for one hour at 42° C. and is gently shaken at intervals, after which it is centrifuged. This supernatant fluid is designated as carbonate extract. With this extract the best results have been obtained, both as regards protection of animals against pneumococcus infection and content of agglutinins and precipitins. In the preparation of the extracts, it is important not to subject the precipitates to prolonged shaking, as this procedure diminishes or destroys their antibody content. Shaklee and Meltzer (12) have previously shown that prolonged shaking destroys the ferments trypsin and pepsin.

EXPERIMENTAL.

Protective Properties of Specific Precipitates and Extracts of Precipitates from Antipneumococcus Serum.

The whole precipitates, and more especially the extracts of the precipitates, protect susceptible animals, rabbits and mice, as well, or almost as well, as the whole antipneumococcus serum.

¶ In Table I, which is a protocol of one of the many protection experiments which have been done upon mice with various specific

TABLE I.*

Protective Experiment with a Fixed Amount of Serum of Type II or Serum Derivatives Formed by Various Methods and Dilutions of a Twenty-Four Hour Bouillon Culture of Pneumococcus of Type II, in Total Volume of 0.5 Cc. The Mixtures Were Injected Intraperitoneally into Mice.

Mouse No.	Protective fluid.	Culture.	Result.
		66.	
1	Control.....	0.00001	D., 30 hrs.
2	".....	0.000001	" 35 "
3	Original serum, 0.2 cc. in 0.5 cc.....	0.2	" 3 days.
4	" " " " " " ".....	0.1	" 24 hrs.
5	" " " " " " ".....	0.01	S. 5 days.
6	" " " " " " ".....	0.001	D., 4½ "
7	Precipitate dissolved in N/10 sodium hydroxide, 0.2 cc. in 0.5 cc.....	0.2	" 10 hrs.
8	Precipitate dissolved in N/10 sodium hydroxide, 0.2 cc. in 0.5 cc.....	0.1	" " "
9	Precipitate dissolved in N/10 sodium hydroxide, 0.2 cc. in 0.5 cc.....	0.01	" 40 "
10	Precipitate dissolved in N/10 sodium hydroxide, 0.2 cc. in 0.5 cc.....	0.001	" " "
11	Whole washed precipitate, 0.2 cc. in 0.5 cc.....	0.2	" 4 days.
12	Whole washed precipitate, 0.2 cc. in 0.5 cc.....	0.1	" 56 hrs.
13	Whole washed precipitate, 0.2 cc. in 0.5 cc.....	0.01	S. 5 days.
14	Whole washed precipitate, 0.2 cc. in 0.5 cc.....	0.001	" " "
15	Whole washed precipitate, 4 times concentrated, 0.2 cc. in 0.5 cc.....	0.2	D., 5 "
16	Whole washed precipitate, 4 times concentrated, 0.2 cc. in 0.5 cc.....	0.1	" 24 hrs.
17	Whole washed precipitate, 4 times concentrated, 0.2 cc. in 0.5 cc.....	0.01	S. 5 days.
18	Whole washed precipitate, 4 times concentrated, 0.2 cc. in 0.5 cc.....	0.001	D., 3 "
19	Carbonate extract, 0.2 cc. in 0.5 cc....	0.2	" 4 "
20	" " " " " " ".....	0.1	S. 5 "
21	" " " " " " ".....	0.01	" " "
22	" " " " " " ".....	0.001	" " "
23	Heat extract, 0.2 cc. in 0.5 cc.....	0.2	D., 56 hrs.
24	" " " " " " ".....	0.1	" 24 "
25	" " " " " " ".....	0.01	S. 5 days.
26	" " " " " " ".....	0.001	" " "

* In the tables D. stands for "died," S. for "survived."

precipitates and extracts of precipitates of both Type I and Type II immune serum, it will be noted that the whole precipitate dissolved in sodic hydrate failed to protect. This result with alkaline solutions of the precipitates has been noted several times. As the injection of the dissolved precipitate itself does not harm the mice, it is presumable that the sodic hydrate has a deleterious effect on the immune substances in the precipitate. Of the mice receiving whole

TABLE II.

Protective Experiment with a Fixed Dose of Culture Plus Decreasing Doses of Serum or Serum Derivatives (Same Lot as Experiment I).

Mouse No.	Protective fluid, total volume 0.5 cc.	Culture, pneumococcus Type II.	Result.
1	Control	cc. 0.00001	D., 44 hrs.
2	"	0.000001	" " "
3	Original serum 0.2 cc.	0.01	S. 5 days.
4	" " 0.15 "	0.01	" " "
5	" " 0.1 "	0.01	" " "
6	" " 0.05 "	0.01	" " "
7	" " 0.01 "	0.01	" " "
8	Whole washed precipitate 0.2 cc.	0.01	" " "
9	" " " 0.15 "	0.01	" " "
10	" " " 0.1 "	0.01	" " "
11	" " " 0.05 "	0.01	" " "
12	" " " 0.01 "	0.01	D., 4 "
13	Carbonate extract 0.2 cc.	0.01	S. 5 "
14	" " 0.15 "	0.01	" " "
15	" " 0.1 "	0.01	" " "
16	" " 0.05 "	0.01	" " "
17	" " 0.01 "	0.01	D., 60 hrs.

serum or whole precipitate, those receiving 0.01 cc. of culture survived, while of those receiving the carbonate extract, those receiving as much as 0.1 cc. of culture survived. In other words, the carbonate extract possessed a higher protective power than the original serum, or the whole precipitate. The group of animals receiving heated extract was not as well protected as the group receiving carbonate extract. This is not a sporadic result of the protective action of carbonate extracts, for many experiments with different lots of serum and various samples of extracts of specific precipitates have yielded approximately the same results. Increasing the dose of whole precipitate by concentration does not increase its potency.

This same result has been noted when using whole serum or concentrated globulin fractions of serum as prepared by the method described by Avery (13). Apparently there is a maximum dose of culture against which it is possible to protect a mouse, and increasing the size of the dose of the protective agent does not increase the protection.

On the other hand, if one infects a series of mice with equal doses of culture and attempts to protect them with decreasing doses of serum or serum derivatives, the results have shown that the protective agent is in excess of the needed amount when 0.2 cc. is used as the standard protective dose. Table II illustrates this point. When mice were infected with doses of 0.01 cc. of culture, the mouse that received 0.01 cc. of original serum was protected as well as the one that received 0.2 cc. of serum. In titrating in this manner the protective value of the serum derivatives made up to original volume of serum, it will be seen that there is some loss in potency of the serum derivatives as compared with the original serum. But this can be explained as due to the inevitable slight loss in material during the manipulations incident to the preparation of the specific precipitates and their extracts.

In these experiments the carbonate extract has consistently shown greater protective qualities than the extracts prepared by simple extraction at room temperature or by heating at 42° C.

Further experiments were carried on to determine whether removal of the precipitate from the serum completely exhausted its protective power, and also whether the precipitate still retained protective power after extraction with sodium carbonate.

From the protocol (Table III) it is evident that the exhausted serum and the salt solution used in washing the precipitate free from serum afforded no protection to mice. Apparently the process of washing the precipitate free from the exhausted serum did not diminish appreciably its protective substances. The first carbonate extract protected well; subsequent extractions of the precipitate showed but little potency. While a single extraction removed most of the protective substances that are dissociable from the whole precipitate, some protective power still existed in the residue precipitate which could not be removed by repeated extractions.

TABLE III.

Experiment to Show the Comparative Protective Value of Serum, Exhausted Serum, Wash Water, and Repeated Extraction. The Mixtures Were Made Up in All Cases to a Constant Volume, 0.5 Cc., and Injected Intraperitoneally into Mice.

Mouse No.	Protective fluid.	Culture II 40.	Result.
1	Control.	cc. 0.00001	D., 42 hrs.
2	"	0.000001	" " "
3	"	Culture I 107 0.000001	" 30 "
4	Original serum, 0.2 cc. in 0.5 cc.	Culture II 40 0.1	" 18 "
5	" " " " " " "	0.01	" " "
6	" " " " " " "	0.001	S. 5 days.
7	" " " " " " "	0.0001	" " "
8	Exhausted " " " " " "	0.1	D., 18 hrs.
9	" " " " " " "	0.01	" 20 "
10	" " " " " " "	0.001	" 18 "
11	" " " " " " "	0.0001	" 42 "
12	2d wash water of whole precipitate twice concentrated, 0.2 cc. in 0.5 cc.	0.1	" 18 "
13	2d wash water of whole precipitate twice concentrated, 0.2 cc. in 0.5 cc.	0.01	" " "
14	2d wash water of whole precipitate twice concentrated, 0.2 cc. in 0.5 cc.	0.001	" 29 "
15	2d wash water of whole precipitate twice concentrated, 0.2 cc. in 0.5 cc.	0.0001	" 42 "
16	Carbonate extract		
	1st extraction, 0.2 cc. in 0.5 cc.	0.1	" 18 "
17	" " " " " " "	0.01	S. 5 days.
18	" " " " " " "	0.001	" " "
19	" " " " " " "	0.0001	" " "
20	Carbonate extract		
	2d extraction, 0.2 cc. in 0.5 cc.	0.1	D., 18 hrs.
21	" " " " " " "	0.01	" 4 days.
22	" " " " " " "	0.001	" 3 "
23	" " " " " " "	0.0001	S. 5 "
24	Residue of whole precipitate after 2 carbonate extractions, 0.2 cc. in 0.5 cc.	0.1	D., 18 hrs.
25	Residue of whole precipitate after 2 carbonate extractions, 0.2 cc. in 0.5 cc.	0.01	" " "
26	Residue of whole precipitate after 2 carbonate extractions, 0.2 cc. in 0.5 cc.	0.001	S. 5 days.
27	Residue of whole precipitate after 2 carbonate extractions, 0.2 cc. in 0.5 cc.	0.0001	" " "
28	1st carbonate extract, 0.2 cc. in 0.5 cc.	Culture I 107 0.1	D., 18 hrs.
29	" " " " " " "	0.01	" " "
30	" " " " " " "	0.001	" 42 "
31	" " " " " " "	0.0001	" 72 "

The carbonate extract and heat extracts contained agglutinins and precipitins, but the titer was lower than that of the original serum. A fresh live culture of pneumococcus was agglutinated by the extracts when made up to the original volume of the serum, but not in dilutions of 1 to 5 or 1 to 10. The whole serum itself, on the other hand, agglutinated in dilutions of 1 to 20 or 1 to 30.

The amount of protein in the carbonate extracts is, of course, much less than in the original serum. The whole serum used in this experiment contained 4.98 per cent protein. After removal of the extract the carbonate extract and the residue precipitate each contained 0.08 per cent protein, or about one-sixtieth of the amount in the original serum. It will be further noted that the protection conferred by the use of such extracts is specific, there being no protection against a pneumococcus infection of Group I by an extract of a precipitate from an antipneumococcus serum of Group II, and *vice versa*.

Experiments like the following have been done to ascertain whether the protective substances in antipneumococcus serum can be removed by live washed cultures of pneumococcus. To 25 cc. of antipneumococcus serum, Type I, were added live washed pneumococci of Type I from 150 cc. of a twenty-four hour broth culture. An immediate precipitation occurred. After twenty-four hours the mixture was centrifuged, the precipitate washed in normal saline and emulsified in 12 cc. of normal salt solution. To this emulsion 0.5 cc. of 1 per cent sodium carbonate was added, and flocculation occurred. The mixture was then heated at 42° C. for one hour, being shaken gently at intervals, and then it was heated to 56° C. for one-half hour. After centrifuging, the supernatant fluid was pipetted off and diluted to the original volume of the serum, and is called carbonate extract (Table IV). The sediment was reemulsified and made up to the original volume of the serum with salt solution and is called sediment of precipitate.

This and similar experiments show that antipneumococcus serum can be exhausted of its antibody content by live cultures. To accomplish this, a relatively much larger quantity of live bacteria must be used to exhaust the serum than when a bacterial extract is employed. The washed precipitate of live agglutinated bacteria, after

TABLE IV.

Protective fluid.	Culture I 106. cc.	Result.
Original serum I, 0.2 cc. in 0.5 cc.	0.1	S. 6 days.
" " " " " " " "	0.01	" " "
" " " " " " " "	0.001	" " "
" " " " " " " "	0.0001	" " "
Exhausted " " " " " " " "	0.001	D., 18 hrs.
" " " " " " " "	0.0001	" 30 "
" " " " " " " "	0.00001	" 48 "
Carbonate extract, " " " " "	0.1	" 18 "
" " " " " " " "	0.01	S. 6 days.
" " " " " " " "	0.001	D., 4 "
" " " " " " " "	0.0001	S. 6 "
Sediment of precipitate, 0.2 cc. in 0.5 cc.	0.1	D., 18 hrs.
" " " " " " " "	0.01	" " "
" " " " " " " "	0.001	S. 6 days.
" " " " " " " "	0.0001	" " "
Control	0.00001	D., 30 "
"	0.000001	" " "

being killed by heating to 56° C. for one-half hour, as well as carbonate extracts therefrom, protects susceptible animals against many times the lethal dose of pneumococcus. They are not as potent as precipitates and extracts of precipitates formed from antipneumococcus serum by acetone-killed bacterial extracts. It will be seen from Table IV that mice are protected fairly well by both the carbonate extract of the precipitate and the residue of the precipitate after removal of the carbonate extract. In no case did the precipitates and extracts of precipitates of antipneumococcus serum, treated with live cultures, protect as well as the original serum. The carbonate extracts from these precipitates contain agglutinins and precipitins as well as protective substances.

The Development of Active and Passive Immunity to Pneumococcus Infection by the Use of Antipneumococcus Serum Derivatives.

The injection of specific serum precipitates and extracts of such precipitates from antipneumococcus serum into mice produces a certain amount of active, as well as passive immunity to pneumo-

coccus infection. This is to be expected, as the specific precipitate itself contains bacterial antigen, and it is presumable that in the extracts of these precipitates a certain amount of antigen as well as antibody goes into solution.

It is well known that the passive immunity conferred on mice by the injection of antipneumococcus serum is of short duration and

TABLE V.

Protective fluid.	Mouse No.	Culture, pneumococcus Type I.*	Result.	Mouse No.	Culture, pneumococcus Type I.†	Result.
Whole serum, 0.2 cc. in 0.5 cc.	1	cc.	D., 20 hrs.	4	cc.	D., 18 hrs.
" " " " " "	2	0.01	S.	5	0.01	" 24 "
" " " " " "	3	0.001	"	6	0.001	" 48 "
Whole precipitate emulsion, 0.2 cc. in 0.5 cc.	7	0.1	D., 20 "	11	0.1	" 18 "
Whole precipitate emulsion, 0.2 cc. in 0.5 cc.	8	0.01	" 24 "	12	0.01	S.
Whole precipitate emulsion, 0.2 cc. in 0.5 cc.	9	0.001	" 44 "	13	0.001	"
Whole precipitate emulsion, 0.2 cc. in 0.5 cc.	10	0.0001	" " "			
Carbonate extract, 0.2 cc. in 0.5 cc.	14	0.1	" 20 "	18	0.1	D., 18 "
Carbonate extract, 0.2 cc. in 0.5 cc.	15	0.01	" " "	19	0.01	" 24 "
Carbonate extract, 0.2 cc. in 0.5 cc.	16	0.001	S.	20	0.001	S.
Carbonate extract, 0.2 cc. in 0.5 cc.	17	0.0001	"	21	0.0001	"
Exhausted serum, 0.2 cc. in 0.5 cc.	22	0.1	D., 20 "	25	0.1	D., 18 "
Exhausted serum, 0.2 cc. in 0.5 cc.	23	0.01	" 26 "	26	0.01	" " "
Exhausted serum, 0.2 cc. in 0.5 cc.	24	0.001	" 44 "	27	0.001	" 2 days.
Exhausted serum, 0.2 cc. in 0.5 cc.				28	0.0001	" " "
Control.	29	0.00001	" 26 "	31	0.00001	" 26 hrs.
" "	30	0.000001	" 44 "	32	0.000001	" 48 "

* These cultures were given 5 days later.

† These cultures were given 10 days later.

usually disappears after a period of seven days. On the other hand, the active immunity response of an animal to the injection of a bacterial antigen does not usually appear until the period of passive

immunity has passed. To determine the presence or absence of passive and active immunity in mice in response to the injection of antipneumococcus serum and serum derivatives, a series of mice were injected intraperitoneally with 0.2 cc. each of (1) antipneumococcus serum, (2) whole precipitate derived from antipneumococcus serum, (3) a carbonate extract of the whole precipitate, and (4) exhausted serum. After intervals of five and ten days, varying dilutions of live virulent cultures were injected intraperitoneally.

Table V shows that considerable passive immunity was conferred on mice by the intraperitoneal injection of antipneumococcus serum, which immunity persisted for at least five days but had disappeared after ten days. On the other hand, the immunity conferred by the carbonate extract persisted up to ten days at least, when the period of passive immunity had presumably passed. This would seem to indicate that some degree of active immunity had been produced by the carbonate extract. The mice treated with exhausted serum showed, as was to be expected, no evidence of either passive or active immunity.

Of the mice treated with whole specific precipitate, all died when infected with pneumococci five days later. It is possible that the early disappearance of passive immunity in this case might be due to a state of lowered resistance caused by the injection of the large amount of bacterial antigen contained in the whole precipitate. After ten days other mice in this same group showed a considerable resistance which must be interpreted as due to active immunity. Experiments have been carried on to learn whether the early loss of passive immunity after the injection of whole precipitate, as mentioned above, might be prevented by preliminary heating of the bacterial extract used for precipitation. Heating the bacterial extract for one hour at 56° C. does not injure its precipitative power. The precipitates formed with such heated extracts, however, showed no differences from those formed from unheated extracts. The passive immunity produced by the injection of such precipitates lasts no longer than that after the injection of precipitates formed with unheated extracts.

TABLE VI.

Protective Experiment with a Fixed Amount of Polyvalent Serum or Serum Derivatives and Dilutions of a Twenty-Four Hour Bouillon Culture of Pneumococci of Type I and Type II in Total Volume of 0.5 Cc. The Mixtures Were Injected Intraperitoneally in Mice.

Polyvalent serum of Types I and II + bacterial extract of Type I = Precipitate I.

Polyvalent serum, after removal of Precipitate I, + bacterial extract of Type II = Precipitate II.

Polyvalent serum + bacterial extract of Type II = Precipitate III.

Polyvalent serum, after removal of Precipitate III, + bacterial extract of Type I = Precipitate IV.

Bacterial extract was added in all cases until the serum was exhausted of the corresponding agglutinins.

All precipitates were suspended in saline with sodium carbonate and heated at 42° C. for one hour, shaking gently. Extracts of each were used for protection tests, diluted to the original volume of serum.

Protective fluid.	Culture I to 8.	Result.	Culture II 42.	Result.
Original polyvalent serum, 0.2 cc. in 0.5 cc.	cc. 0.1 0.01 0.001 0.0001	S. 6 days " " " " " " " " "	cc. 0.1 0.01 0.001 0.0001	S. 6 days. D., 18 hrs. S. 6 days. " " "
Extract of Precipitate I, 0.2 cc. in 0.5 cc.	0.1 0.01 0.001 0.0001	D., 18 hrs. " 3 days S. 6 " " " "	0.1 0.01 0.001 0.0001	D., 18 hrs. " 30 " " 18 " " 30 "
Extract of Precipitate II, 0.2 cc. in 0.5 cc.	0.1 0.01 0.001 0.0001	D., 18 hrs. " " " " 30 " " 42 "	0.1 0.01 0.001 0.0001	" 18 " " 30 " " 30 " S. 6 days.
Extract of Precipitate III, 0.2 cc. in 0.5 cc.	0.1 0.01 0.001 0.0001	" 18 " " " " " " " S. 6 days	0.1 0.01 0.001 0.0001	D., 18 hrs. " 56 " S. 6 days. " " "
Extract of Precipitate IV, 0.2 cc. in 0.5 cc.	0.1 0.01 0.001 0.0001	D., 18 hrs. " 24 " S. 6 days D., 3 "	0.1 0.01 0.001 0.0001	D., 18 hrs. " " " " " " " 24 "
Controls.	0.00001 0.000001	" 24 hrs. " " "	0.00001 0.000001	" 18 " " " "

TABLE VII.

The Specificity of Agglutinins Contained in Extracts of Specific Precipitates from Polyvalent Antipneumococcus Serum. The Extracts Are Obtained by Extracting the Specific Precipitates (Experiment VI) in Saline with Weak Sodium Carbonate + Heat.

Polyvalent serum + bacterial extract of Type I = Precipitate I.

Polyvalent serum, after removal of Precipitate I, + bacterial extract of Type II = Precipitate II.

Polyvalent serum + bacterial extract of Type II = Precipitate III.

Polyvalent serum, after removal of Precipitate III, + bacterial extract of Type I = Precipitate IV.

Original polyvalent serum dilution.	+ Culture, pneumococcus Type I.		Original polyvalent serum dilution.	+ Culture, pneumococcus Type II.	
	2 hrs.	24 hrs.		2 hrs.	24 hrs.
I : I	++	++	I : I	++	++
I : 5	++	++	I : 5	++	++
I : 10	+	+	I : 10	+	+
I : 20	-	-	I : 20	-	-
Extract of Precipitate I + Culture I			Extract of Precipitate I + Culture II		
5 : I	++	++	I : I	-	-
I : I	-	-	I : 5	-	-
I : 2.5	-	-	I : 10	-	-
I : 5	-	-	I : 20	-	-
Extract of Precipitate II + Culture I			Extract of Precipitate II + Culture II		
5 : I	-	-	5 : I	++	++
I : I	-	-	I : I	-	-
I : 2.5	-	-	I : 2.5	-	-
I : 5	-	-	I : 5	-	-
Extract of Precipitate III + Culture I			Extract of Precipitate III + Culture II		
5 : I	-	-	5 : I	++	++
I : I	-	-	I : I	-	-
I : 2.5	-	-	I : 2.5	-	-
I : 5	-	-	I : 5	-	-
Extract of Precipitate IV + Culture I			Extract of Precipitate IV + Culture II		
5 : I	++	++	5 : I	-	-
I : I	-	-	I : I	-	-
I : 2.5	-	-	I : 2.5	-	-
I : 5	-	-	I : 5	-	-

All agglutination tests are done by the macroscopic method, 0.3 cc. of twenty-four hour broth culture + 0.3 cc. of the serum or extract dilution being used. Readings are made after 2 hours in the water bath at 38° C. and after 24 hours on ice. If agglutination occurs it is usually apparent in 5 minutes.

TABLE VIII.

The Specific Adsorption of Agglutinins from a Polyvalent Pneumococcus Immune Serum by Use of Live Washed Cultures of the Pneumococcus and the Dissociation of These Agglutinins from the Specific Precipitates. Live Washed Cultures of Pneumococci of Type I and Type II Were Used in Obtaining the Precipitates and Extracts from a Polyvalent Antipneumococcus Serum.

Polyvalent serum + live culture of pneumococcus Type I = Precipitate I.

Polyvalent serum, after removal of Precipitate I, + live culture of pneumococcus Type II = Precipitate II.

Polyvalent serum + live culture of pneumococcus Type II = Precipitate III.

Polyvalent serum, after removal of Precipitate III, + live culture of pneumococcus Type I = Precipitate IV.

All precipitates were heated for one-half hour at 56° C., and extracted with saline + carbonate.

The extracts were used for agglutination tests.

Dilution of serum.	Culture I.		Dilution of serum.	Culture II.	
	2 hrs.	24 hrs.		2 hrs.	24 hrs.

Polyvalent serum after removal of Precipitate I by live culture of pneumococcus Type I.

I : I	—	—	I : I	+	+
I : 5	—	—	I : 5	—	—
I : 10	—	—	I : 10	—	—
I : 20	—	—	I : 20	—	—

Polyvalent serum after removal of Precipitate III by live culture of pneumococcus Type II.

I : I	+	+	I : I	—	—
I : 5	—	+	I : 5	—	—
I : 10	—	—	I : 10	—	—
I : 20	—	—	I : 20	—	—

Extract I.			Extract II.		
	2 hrs.	24 hrs.		2 hrs.	24 hrs.
Culture I.	+	+	Culture I.	—	—
" II.	—	—	" II.	—	+

Extract III.			Extract IV.		
	2 hrs.	24 hrs.		2 hrs.	24 hrs.
Culture I.	—	—	Culture I.	+	+
" II.	+	+	" II.	—	—

The Specific Adsorption of Immune Substances from Polyvalent Serum by Bacterial Extracts and Live Bacteria.

A polyvalent antipneumococcus serum may be specifically exhausted of its immune bodies for one of the types of pneumococcus by the addition of a bacterial extract of the corresponding type. The immune substances of the other type remain intact and can be removed subsequently by the addition of the appropriate antigen (Tables VI and VII). During the process of fractional precipitation the titer of the immune bodies is somewhat diminished. This diminution may be accounted for partly by the loss of precipitate incident to the manipulation in washing, and partly by the supposition that the antigen-antibody combination is only partially dissociated. Table VIII shows that the specific agglutinins may be removed in like manner from the polyvalent serum by the use of living pneumococci in place of bacterial extracts.

Nature of the Union between Precipitin and Precipitinogen.

In the above detailed experiments it has been shown that the protective substances in antipneumococcus serum can be removed specifically by precipitation with an extract of the homologous type of pneumococcus, and that this precipitate, when suspended in salt solution and injected into susceptible animals, will protect them against many times the lethal dose of pneumococcus.

It has been further shown that the union between the bacterial extract (precipitinogen) and the immune substances in the serum can be to some extent disunited by suitable chemical and physical agents.

The following observations which we have made suggest strongly that in the formation of the precipitate an actual union occurs between the precipitin of the serum and precipitinogen of the bacterial extract. If, before adding the precipitinogen to the serum, the latter be heated at 60° C. for an hour, no precipitation takes place. Nor can the precipitin be reactivated by the addition of unheated normal serum. This fact, that precipitins are inactivated by heat, and then cannot be reactivated by fresh serum, has already been demonstrated by Pick (14) and Kraus and von Pirquet (15). That

a union, however, has occurred between the precipitin and precipitinogen, even in the absence of precipitate, is made evident by the fact that if to a mixture of bacterial extract and heated immune serum fresh unheated immune serum be added, no precipitate occurs. The most likely explanation is that the precipitable substance is already saturated with the inactivated precipitins.

By suitable extraction methods as shown above, an apparent dissociation of antigen and antibody occurs. It seems likely, however, that, although a dissociation has occurred, and the agglutinins and protective substances have been set free, a permanent change has occurred in the precipitinogen, for when fresh immune serum is added to the extracts of the specific precipitates, even if the latter be concentrated, no fresh precipitate forms.

DISCUSSION.

These studies suggest that the use of precipitate extracts, prepared as described, may offer certain advantages over the use of whole serum in the treatment of lobar pneumonia, since the extracts possess practically the entire immunizing and protective power of immune serum, and yet contain a very small fraction of the serum proteins. The patient may thus be relieved of the strain incident to the metabolism of the large amounts of protein contained in the large quantities of serum which it is now necessary to employ. It is altogether probable, moreover, that the symptoms of serum sickness, which so frequently follow the use of large amounts of serum, may be lessened, if not prevented entirely, by the use of precipitate extracts. The precipitates and extracts of precipitates are still able to produce the phenomena of anaphylaxis and serum sickness, however, as is shown by the fact that in guinea pigs sensitized to horse serum, acute anaphylactic death may be induced by the injection of precipitates or precipitate extracts. To produce this phenomenon, however, fairly large amounts of these substances are required.

The extracts of precipitates, moreover, may have an additional advantage over serum alone in treatment, in that, in addition to conferring passive immunity, they also induce the production of

some degree of active immunity. While the active immunity becomes evident at so late a period that in acute lobar pneumonia it could not be of much therapeutic importance, yet in other, more chronic infections, a similar active reaction induced by suitable extracts might prove of value.

• CONCLUSIONS.

1. The protective substances contained in specific precipitates from antipneumococcus serum can be extracted by suitable chemical and physical agents, dilute sodium carbonate at 42° C. being especially advantageous as an extractive agent.

2. The resulting water-clear extracts, when made up to the original volume of the serum used for precipitation, protect animals almost as well as does the whole serum.

3. The bacterial extracts used in precipitating the protective substances from the serum act specifically; that is, a bacterial extract of pneumococcus of Type I removes the protective substances from a Type I immune serum only.

4. In a polyvalent serum of Type I and Type II, the protective substances of each type may be removed independently of each other by the successive addition of the homologous antigens.

5. Extracts of specific serum precipitates contain only one-fiftieth to one-sixtieth of the protein in the original serum, and about one-half the protein of the whole precipitate.

6. Extracts contain not only protective substances but agglutinins and precipitins.

7. Extracts and whole precipitates not only confer passive immunity but stimulate the production of active immunity to pneumococcus infection in rabbits and mice.

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